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14. ABSTRACT While many advancements have been made in breast cancer therapy, metastatic breast cancer remains an incurable disease. MUC1 is a glycoprotein expressed on normal glandular epithelial but is over-expressed and underglycosylated in over 90% of human breast tumors and metastatic lesions, which lead to its ranking by NCI as the second most targetable antigen. To this end, we have proposed vaccinating with peptides from the MUC1 protein core, which is only visible to the immune system on the tumor-associated form of the protein. Previous work from our lab has demonstrated that this vaccine does elicit a MUC1-specific immune response that can only be functional if the immunosuppressive tumor microenvironment is altered to allow efficient killing of tumor cells. Thus, we investigated the effectiveness of MUC1 vaccination in combination with drugs known to inhibit immunosuppression to determine which drug is the most effective. Mice that are transgenic for human MUC1 (MUC1.Tg) mice were orthotopically injected with a syngenic breast cancer cell line expressing human MUC1 (Mtag.MUC1). Mice were vaccinated after palpable tumor formation three times at a 10 day interval with the vaccine cocktail, consisting of two MHC class I-restricted MUC1 tandem repeat peptides and a class II pan helper peptide mixed with GM-CSF and CpG ODN, in incomplete Freund's adjuvant. Previous work in our lab has shown that blocking the cyclooxygenase pathway (COX) resulted in an inhibition of immunosuppression. Thus we used the following drugs in combination with the MUC1-vaccine therapy: Indomethacin (COX1 and COX2 inhibitor), Celecoxib (COX2 inhibitor), 1-methyl tryptophan (indoleamine 2,3 dioxygenase inhibitor), and AH6809 (EP2 receptor antagonist). Mice were euthanized and tissue was collected 2 days post the final vaccination. Immune activation was measured via an ELISPOT for IFN- γ using T cells from either tumor-draining lymph nodes or splenocytes as responders and dendritic cells pulsed with the vaccinating peptides as simulators. MUC1 vaccine therapy alone causes a slight reduction in tumor burden, although not significant, but does induce IFN- γ production from T cells, indicating immune cell activation. The combinational therapy of Indomethacin + Vaccine results in a significant reduction in tumor size. All drugs cause significantly higher levels of IFN- γ production from T cells compared to vaccine alone. Treatment with both Indomethacin and Celecoxib reduce PGE2 levels compared to vaccine alone. In a repeat experiment, we found that vaccine alone and indomethacin alone caused a slight reduction in tumor burden, but tumor burden was only significantly reduced when the therapy was given in combination. In this model, MUC1 vaccine therapy can be enhanced when given in combination with Indomethacin, this was correlated with an increase in TUNEL positive cells in the full treatment group as well as alterations in expression of OPG, arginase, and

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INTRODUCTION:

Breast cancer is the second most common cancer in the United States and contributes to 40,000 deaths a year. Research now focuses on the development of novel breast cancer-specific vaccines. MUC1 is a transmembrane mucin glycoprotein that is overexpressed in >90% of breast carcinomas [7-11]. Recently, MUC1 was listed as the second most targetable tumor antigen by the national cancer institute [4]. Our lab has demonstrated the effectiveness of MUC1-directed tumor vaccines in colorectal, pancreatic, and breast cancer models; however immunosuppression was observed at the tumor site, hindering the immune response to the vaccine [2, 12, 13]. Thus, combining immunotherapy with available adjuvant treatments may sufficiently alter the tumor microenvironment such that the effector cells can function properly. COX-2 is an enzyme that converts arachidonic acid to prostaglandins. COX-2 is induced in breast cancer during various pathologic conditions. Our lab previously found that Cyclooxygenase 2 (COX2) over-expression and subsequent Prostaglandin E2 (PGE2) production, in response to vaccination, are immunosuppressive [2, 14]. Further, COX-2 inhibition, via the use of Celecoxib, reduced breast tumor levels of indoleamine 2, 3-dioxygenase (IDO). This project is focused on 1) understanding the role of IDO enzymatic activity on tumor development and immune function and 2) investigating the efficacy of a MUC1-based vaccine in combination with a variety of targeted inhibition of immune suppression in an effort to achieve a maximum clinical response and 3) Determining the mechanism behind the enhanced efficacy. In previous years, we concluded that tumor burden does not differ between tumors that were injected into IDO null mice or blk6 mice, no matter whether they were IDO expressing or IDO null tumors. As far as tumor burden is concerned, the phenotype of the mouse does not matter, but the phenotype of the tumor does. This is demonstrated by IDO null tumors that have significantly lower tumor burden than either of the two IDO producing tumors. Moreover, we tested the MUC1 specific tumor vaccine with targeted inhibition of immune suppression in an effort to achieve a maximum clinical response. We tested the MUC1 vaccine in combination with an indoleamine 2,3 dioxygenase (IDO) inhibitor (1-MT; 1-methyl tryptophan), a COX1 and COX2 inhibitor (Indomethacin), a COX2 inhibitor (Celecoxib), as well as in combination a PGE2 antagonist (AH6809). Our results indicate that Indomethacin in combination with the MUC1 vaccine resulted in a significant reduction in tumor burden. All other drug combinations tested were unable to significantly reduce tumor burden at the dosages tested. This year, we focused on determining the mechanism behind the enhanced efficacy.

BODY:

Previous Progress Years 1 & 2

In order to address the first aim of our project, to determine the effects of IDO deletion on tumor development and immune functions in a mouse model of spontaneous mammary gland tumors, we needed to determine which factor was more important for immune function: IDO in the tumor microenvironment, or IDO presence in the body. Therefore to appropriately assess this, we injected indoleamine 2,3-dioxygenase knockout (IDO^{-/-}) mice as well as C57 black6 mice with multiple breast cancer tumors. The tumors were injected into the mammary fat pad of 6-8 week old mice. We injected multiple cell lines to produce tumors 1) IDO expressing PyVMT (also known as MTAG) cells, 2) IDO null PyVMT (also known as IKOM) cells and 3) IDO expressing MTAG.MUC1 cells. Therefore, we had mice that 1) expressed IDO in the tumor, but not in the body 2) did not express IDO in the tumor or in the body 3) expressed MUC1, expressed IDO in the tumor, but not in the body 4) expressed IDO in the tumor, and in the body 5) did not express IDO in the tumor but did express IDO in the body 6) expressed MUC1, expressed IDO in the tumor, and in the body (n=3 each). The MUC1 expressing tumors were included in this part of the study, as they will later be the target of the MUC1 based vaccine. These tumors were allowed to grow for 38 day in vivo.

Previously, the PyVMT (Polyoma virus Middle T Antigen) spontaneous breast cancer mouse model was tested in our lab. PyVMT tumors from untreated mice were dissected and dissociated using collagenase IV. The cell line generated from these tumors was designated as MTAG cells. In order to test the MUC1 vaccine in vivo, in an injectable breast cancer model, we transfected the MTAG cells with the full length MUC1 plasmid. In order to insure a high purity of MUC1 expressing MTAG.MUC1 cells, the transfected cell line was sorted for MUC1 expression using FacsAria. Expression phenotype of the MUC1 cell line was analyzed using the HMFG2 antibody which targets sparsely glycosylated VNTR repeats of the human MUC1 extracellular domain. Using HMFG2 antibodies for flow cytometry, we confirmed that MTAG.MUC1 cells are positive for MUC1 (Figure 9).

Tumors were palpated every other day starting at day 10 post injection when tumors became palpable. Animal health was observed daily for any sign of distress such as weight loss greater than 10% of starting body weight, hind limb paralysis, failure to eat or drink, hunching or lethargy. The mammary gland tumors were dissected, weighed, and part was prepared as lysate for future analysis and part of it was paraffin embedded for sectioning immunohistochemistry (IHC) to evaluate apoptosis and proliferation *in situ*, and infiltrating immune cells. Lungs were dissected and examined for metastasis by gross examination and paraffin embedded for future sectioning. At time of sacrifice, serum was collected. In the future, we will use a cytokine array kit to determine levels of TH1, TH2, And TH17 response. Moreover, Since PGE2 has been shown to regulate IDO function, we will detect PGE2 levels using specific ELISA, and kynurine and tryptophan by modified HPLC in tumor lysates and serum.

In the IDO^{-/-} mice, MTAG and MTAG.MUC1 tumors grew rapidly, whereas the IKOM tumors were rejected (Figure 1). MTAG and MTAG.MUC1 tumors were significantly larger than IKOM tumors from days 26-day 38 post injection ($p < 0.01$). Indicating that, even in an IDO null body, IDO presence in the tumor seems to be an important factor in tumor progression. In the Blk6 mice, MTAG and MTAG.MUC1 tumors grew rapidly, and again, IKOM tumors were rejected (Figure 2). MTAG and MTAG.MUC1 tumors were significantly larger than IKOM tumors from days 26-day 38 post injection ($p < 0.01$). This again indicates that, IDO in the tumor seems to be an important aspect for tumor development, even when the body expresses IDO. This data is recapitulated in the tumor wet weight, as seen in Figure 3. The tumor wet weight of MTAG tumors was significantly higher in the black6 mice as compared to both IKOM and MTAG.MUC1 tumors also injected into blk6 mice ($p < 0.001$). Although there is a trend in which MTAG tumor size is decreased when injected into IDO^{-/-} mice, there is no statistical significance. The tumor wet weight in the IDO^{-/-} mice is similar in that

MTAG tumor burden was significantly higher than MTAG.MUC1 and IKOM tumors. IKOM tumor burden, in both cases was significantly smaller than the IDO expressing tumors, MTAG, and MTAG.MUC1 ($p < 0.01$).

When comparing IDO expressing-MTAG tumors injected into blk6 or IDO^{-/-} mice, there was no statistical significance in tumor burden, as assessed by caliper measurements (Figure 4). IDO expressing MTAG.MUC1 tumors injected into blk6 or IDO^{-/-} mice were not statistically different, as shown in Figure 5. The IDO null cells injected into blk6 and IDO^{-/-} were also not statistically different, although it is interesting to note that for the IKOM tumor cells, the tumors were starting to develop and progressed well until day 24, at which point, the NK cells, or T cells were able to clear the tumor, and reduce tumor burden to nothing (Figure 6). This exemplifies the importance of IDO in the tumor, in order for the immune system to appropriately clear the tumor.

At the time of sacrifice, splenocytes were collected and stained for T regulatory cells and myeloid-derived suppressor cells. Tregs play a key role in the maintenance of immune tolerance to both self-and foreign antigens [15, 16]. Upon antigen stimulation, Tregs potently suppresses the activation/proliferation of CD4⁺ or CD8⁺ cells *in vitro* [17]. It is well established that Tregs are present in the tumor microenvironment and hamper efficient anti-tumor immune responses [18, 19]. Several reports have documented the potential role of Treg removal for the induction of tumor rejection.

Although Tregs are well known as suppressor cells there are other types of suppressor cells like MSCs, also known as immature myeloid cells, IMC or M2-macrophages [20-22]. MSCs can suppress the activation of CD4⁺ and CD8⁺ T cells, inhibiting the generation of an antitumor response [23-25]. MSCs are thought to be induced by a variety of cytokines and growth factors (TGF- β , VEGF) which are produced within the tumor microenvironment [26, 27]. MSCs have poor antigen-presenting capability, and produce factors that suppress T cell proliferation and activity, and promote angiogenesis [28].

Two and three-color flow cytometry was used to assess Tregs and MDSCs in the IDO^{-/-} and blk6 mice. Flow cytometry antibodies included: for Tregs, APC-labeled anti-FoxP3 (ebiosciences, San Diego CA, clone FJK-16s), PE-labeled anti-CD25 (Pharmingen, San Diego, CA, clone pc-61), and FITC-labeled anti-CD4 (Pharmingen, clone GK1.5); for MSCs, FITC-labeled anti-CD11b (Pharmingen, clone M1/70) and PE-labeled anti-Gr1 (Pharmingen, clone RB6-8c5). Cells were acquired on a flow cytometer and analyzed with BD Biosciences FlowJo version 8. Tregs (CD4⁺FOXP3⁺) will also be assessed by IHC on the tumor sections.

Figure 7 shows the percentage of CD4⁺, CD25⁺, FoxP3⁺ Splenocytes (Tregs), in each of the IDO^{-/-} and blk6 injected mice. The IDO^{-/-} mice injected with tumor cells had significantly lower percentages of Tregs than the blk6 mice injected with the same tumor cells ($p < 0.05$). However, there was no statistical difference between the tumor cells injected. Thus indicating, that for percentage of T regulatory cells, IDO presence in the body seems to be more important than IDO presence in the tumor. The same does not hold true for the percentage of myeloid derived suppressor cells however. The percentage of MDSCs are significantly higher when IKOM cells are injected into blk6 as compared to IDO^{-/-} mice (Figure 8). Interestingly, the percentage of MDSCs were significantly higher when MTAG cells were injected into IDO^{-/-} mice. MDSCs from MTAG.MUC1 injected mice remained unchanged regardless of the background. However, this is simply a phenotypic analysis of the MDSCs, not a functional analysis. Therefore, in the future, further analysis of the MDSCs including a functional analysis, as well as a subset analysis will be crucial to fully elucidating the effect of IDO on MDSCs.

The second aim of this project is to: To test the MUC1 vaccine in combination with a variety of targeted inhibition of immune suppression in an effort to achieve a maximum clinical response. This included testing the combination of vaccine in combination with an indoleamine 2,3 dioxygenase (IDO) inhibitor (1-MT; 1-methyl tryptophan), a COX1 and COX2 inhibitor (Indomethacin), a COX2 inhibitor (Celecoxib), as well as in combination a PGE2 antagonist (AH6809). In order to test the efficacy of the vaccine in combination mice were orthotopically injected in the mammary fat pad. 24 female MUC1.Tg mice were orthotopically injected with MTAG.MUC1

cells in the mammary fat pad. When tumors were palpable, approximately day 8 post tumor cell injection (p.t.i.), mice were randomly assigned to five different treatment groups: vaccine alone, vaccine + 1-MT, vaccine + indomethacin, vaccine + celecoxib, vaccine + AH6809. Unfortunately, in this pilot experiment, we did not have MUC1.Tg female mice available to include all appropriate controls. In future experiments, this pilot experiment will be repeated with the appropriate controls included.

All treatment groups were administered the MUC1 vaccine subcutaneously on day 8 p.t.i. In addition to vaccine administration, mice were treated with either 1-MT (400mg/kg), indomethacin (3mg/kg), Celecoxib (10mg/kg), or AH6809 (200ug) on a five day on, two day off, schedule. All drugs were administered once per day with the exception of 1-MT which was administered twice per day. Mice were again administered the MUC1 vaccine on days 19, 34 and 35 p.t.i. Mice were monitored for signs of distress, and tumor burden was measured three times per week. Mice were sacrificed on day 35 p.t.i. Results demonstrate that MTAG.MUC1 tumors treated with a combination of vaccine + indomethacin significantly reduced tumor burden beginning on day 30 p.t.i. as compared to vaccine alone. This significance was maintained until mice were sacrificed (Figure 10). All other treatment combinations did not display a significant reduction in tumor burden compared to vaccine alone. Upon sacrifice, the tumors were weighed, prepared for lysates, and fixed for immunohistochemistry. Analysis of the tumor wet weight displayed similar trends, suggesting that the only group in which there was a reduced tumor wet weight was the vaccine + indomethacin group, however, this reduction was not significant (Figure 11).

COX-2 derived PGE-2 is the major prostaglandin produced by breast cancer cells. Production of PGE2 in the tumor lysate is an appropriate measure of COX-2 activity in this orthotopic mouse model of breast cancer; however, PGE2 is unstable *in vivo*. Therefore, we measured PGEM, the PGE2 metabolite (namely, 13,14-dihydro-15-keto-PGA2) in order to provide a reliable estimate of PGE2 production. PGEM levels were measured in the tumor lysates of all treatment groups by ELISA. A significant reduction in tumor PGEM was observed in mice treated with vaccine + celecoxib, as well as vaccine + indomethacin, as compared to vaccine alone ($p < 0.05$, Figure 12). There was no significant reduction of PGEM levels of mice treated with the combination of vaccine + 1-MT.

As stated previously, COX-2, PGE2, and IDO have been linked with T regulator (T-regs) and myeloid-derived suppressor cells (MDSCs) presence in the tumor microenvironment. Tregs play a key role in the maintenance of immune tolerance to both self-and foreign antigens and are reviewed in [15]. Upon antigen stimulation, Tregs potently suppresses the activation/proliferation of CD4⁺ or CD8⁺ cells *in vitro*. It is well established that Tregs are present in the tumor microenvironment and hamper efficient anti-tumor immune responses. Several reports have documented the potential role of Treg removal for the induction of tumor rejection. Although Tregs are well known as suppressor cells there are other types of suppressor cells like MDSCs, also known as immature myeloid cells [20-22]. MDSCs can suppress the activation of CD4⁺ and CD8⁺ T cells, inhibiting the generation of an antitumor response [23-25, 29, 30]. MDSCs are thought to be induced by a variety of cytokines and growth factors (TGF- β , VEGF) which are produced within the tumor microenvironment [26, 27]. MDSCs have poor antigen-presenting capability, and produce factors that suppress T cell proliferation and activity, and promote angiogenesis [28]. This phenotype contrasts markedly with the phenotype of classically activated type I or M1 macrophages that are efficient immune effector cells able to kill microorganisms and tumor cells, present antigens, and produce high levels of T cell stimulatory cytokines.

Therefore, in order to determine the underlying mechanism of the inefficacy of the vaccine + 1-MT treatment, we isolated splenocytes from MTAG.MUC1 tumors bearing mice, pooled the splenocytes, stained, and assessed a number of immune parameters. Levels of myeloid-derived suppressor cells were assessed, characterized by the co-expression of Gr1 and CD11b. There was no significant difference observed in MDSC levels in mice treated with any of the combinational treatments tested (Figure 13A). Helper T cells were defined as CD4⁺, whereas T regulatory cells (Tregs) were characterized by the coexpression of CD4 and FoxP3. No significant difference was observed in the percentage of helper T cells or Tregs in any of the combinational treatments tested (Figure 13 B, C). However, there was a slight increase in the percentage of Tregs in the mice treated with the combination of vaccine +AH6809, although this increase was not significant.

Functionally distinct phenotypes of CD8⁺ T cells spanning from naïve (CD8⁺CD62L⁺CD11b⁻CD44⁻) to an effector and/or memory stage of differentiation have been described [31]. Effector CD8⁺ T cells (CD8⁺CD62L⁻CD11b⁺CD44⁺), are terminally differentiated and are known to release an array of cytokines upon stimulation (IFN- γ and TNF- α), as well as display strong cytolytic activity with high expression of perforin and granzyme. Memory T cells were defined as CD8⁺CD62L⁻CD11b⁻CD44⁺. Therefore, in order to determine the nature of the cells induced by this treatment, we assessed levels of naïve, memory and effector T cells, as well as CD8⁺ T cells. No significant differences were observed among the different treatment groups in overall CD8⁺ T cells (Figure 14 A). The Naïve T cell population was significantly reduced in the vaccine + celecoxib treatment group (Figure 14 B). The combinational treatment of vaccine + AH6809 significantly reduced effector T cell populations (Figure 14C), while there was no significant difference observed among any of the combinational treatment groups with respect to memory T cells (Figure 14D).

In order to examine the growth inhibitory effect that these drugs have on the tumor cells in vitro, MTAG.MUC1 tumor cells were treated with each drug and its corresponding vehicle control. Cells were treated following 24 hours of serum starvation to achieve cell cycle synchronization. Cells were treated with doses of drug ranging from 0uM to 400uM. Proliferation was measured by [³H]-thymidine uptake at 24 and 48 hours post treatment. Celecoxib treatment resulted in a significant decrease in proliferation at all dosages tested at both 24 and 48 hours post treatment (Figure 15A, Figure 16A). MTAG.MUC1 cells treated with AH6809 showed no significant decrease in proliferation compared to vehicle control, irrespective of the dose given or time point tested (Figure 15B, Figure 16B). It appears as though the vehicle used for administering AH6809 may be toxic to the cells itself, and therefore needs to be optimized before conclusions can be drawn about the effect of AH6809 on MTAG.MUC1 cells. Indomethacin treatment resulted in a significant decrease in proliferation when treated with dosages ranging from 100-400uM, at both 24 and 48 hours post treatment (Figure 15C, Figure 16C). Additionally, at 24 hours post treatment, there was a significant decrease in proliferation when MTAG.MUC1 cells were treated with 50uM of Indomethacin (Figure 15C). No significant difference was observed when cells were treated with varying doses of 1-MT, at both 24 and 48 hours post treatment (Figure 15D, Figure 16D). Again, the variability in this data suggests that the vehicle needs to be optimized for 1-MT administration.

In order to further examine the enhanced efficacy of the combinational treatment vaccine + indomethacin, since we were not seeing any enhanced efficacy with the combinatorial 1-MT treatments, female MUC1.Tg mice were orthotopically injected with MTAG.MUC1 cells in the mammary fat pad. By day 6 p.t.i. tumors were palpable, and mice were divided into four different treatment groups. One group served as a control, whereas the other three groups were treated with indomethacin alone, vaccine alone, or vaccine + indomethacin. The treatment groups receiving the MUC1 vaccine were vaccinated on days 6, 15, 24, 27, and 28 p.t.i. Mice receiving indomethacin treatment were gavaged three days per week (3mg/kg). Tumor burden was monitored three times per week, while body weight was measured twice weekly. Mice were sacrificed on days 27 and 28 p.t.i. Results demonstrate that MTAG.MUC1 tumors treated with the combination of vaccine + indomethacin resulted in a significantly reduced tumor burden beginning at day 17. This significant reduction in tumor burden was maintained until mice were sacrificed (Figure 17). Indomethacin alone, as well as vaccine alone, resulted in a significant reduction in tumor burden, as compared to control, beginning at 24 days p.t.i (Figure 17B). Results also demonstrated that tumor burden of mice treated with vaccine + indomethacin was significantly lower than either indomethacin alone or vaccine alone. This significance was noted at day 20 p.t.i and remained until mice were sacrificed (Figure 17B). This is suggestive of a synergistic effect between vaccine and indomethacin treatment.

Upon sacrifice, the tumors were weighed, prepared for lysates, and fixed for immunohistochemistry. Analysis of the tumor wet weight displayed similar trends, specifically, mice receiving the combination treatment of vaccine + indomethacin had significantly decreased tumor wet weight as compared to control ($p < 0.01$). Moreover, the combination treatment also resulted in a significantly reduced tumor burden compared to vaccine alone ($p < 0.05$, Figure 17). However, no significant difference was observed between mice treated with indomethacin alone and control mice (Figure 18). In order to insure that the treatment was indeed effective in reducing PGE2 levels, Prostaglandin E2 Metabolite (PGEM) was again measured in the tumor lysate of

treated mice as a read out for PGE2 levels. The combination of vaccine + indomethacin as well as indomethacin alone, significantly decreased levels of PGEM in the tumor lysate of treated mice as compared to control mice (Figure 19). Additionally, the mice treated with the combination treatment of vaccine + indomethacin resulted in significantly decreased PGEM levels as compared to vaccine alone ($p < 0.05$, Figure 19). Thus, we believe that this combinational treatment is immunologically relevant and warrants further investigation.

Current Progress

Aim 3 of this project was to determine in the MUC1.Tg mice, the mechanism associated with the enhanced vaccine efficacy in combination with Indomethacin. Figure 20 confirms MUC1 expression via western blot analysis as well as IHC, in addition to COX expression in these tumors. We show immunological and tumor analysis of mice treated with the MUC1 peptide vaccine and Indomethacin in Figure 21. At this point, we had not been able to observe effective killing of tumor cells by lymphocytes isolated from vaccinated mice *ex vivo*. Thus we hypothesized that pretreatment of the tumor cells with indomethacin would render them sensitive to lymphocyte killing. To test this hypothesis we first vaccinated tumor bearing mice with the MUC1 peptide vaccine and isolated effector cells from the tumor-draining lymph nodes of these mice. Separately we treated Mtag.MUC1 cells (target cells) with increasing doses of the vehicle control or indomethacin. After 24hrs, the media was discarded and cells were washed to remove any remaining indomethacin. Then effector cells were added at 50:1 and 25:1 effector:target ratios, and the ability of effector cells to impede tumor cell proliferation was assessed by the addition of tritiated thymidine for 24hrs. Again the plates were then washed to remove the lymphocytes, and then thymidine incorporated into the Mtag.MUC1 tumor cells was evaluated by using the Topcount microscintillation counter. At the 400 μ M dose of Indomethacin, Mtag.MUC1 proliferation was significantly inhibited by effector cells (Figure 22A). To further investigate if tumor cell killing was occurring, we performed chromium release assay using the same effector cells isolated from tumor bearing mice vaccinated with the MUC1 peptide vaccine. The target cells were Mtag.MUC1 tumor cells pretreated with 25 and 100 μ M of indomethacin for 24hrs and then labeled with tritiated thymidine and plated with effector:target ratio of 100:1-3.25:1. At the highest effector:target ratio, we were able to observe effective tumor cell killing only when the Mtag.MUC1 tumor cells were pre-treated with Indomethacin at the high dose (Figure 22B), indicating that Indomethacin treated allows for direct killing of the tumors cells by immune cells.

To test if these *in vitro* effects were occurring *in vivo*, we investigated the effects of Indomethacin on tumor cell proliferation and apoptosis in tumor lysate from our mice (Figure 23). We observed no difference in PCNA expression in our treatment groups (Figure 24A-B). Further, we did not observe a difference in phospho-ERK or phospho AKT levels in the treated mice (Figure 24C-F). Tunel stain showed a trend of increase in the vaccine and vaccine + indo groups compared to control, but full treatment showed a significant increase in Tunel positive cells, indicating that more apoptosis is occurring in the full treatment mice compared to control mice (Figure 23A-B). We assessed levels of apoptotic markers but observed no changes were observed in the anti-apoptotic protein, Bcl-2, or the pro-apoptotic, Bax or Caspase-9, expression in the tumors (Figure 23C-F).

Next we conducted microarray analysis of RNA isolated from the tumors of mice. The top ten significantly up-regulated and down-regulated genes from the treatment groups compared to untreated mice are displayed in Figure 25. Interestingly many factors related to the immune system activation, such as S100A8, S100A9, Fc receptors, MHC Class II molecules and even arginase were significantly up-regulated. Osteoprotegerin (Tnfrsf11b) was significantly down-regulated, which is an inhibitor of acquired tumor cell killing. We attempted confirm these findings with Western blot analysis of tumor lysates from treated mice. CCN1 (Cyr61), which is known to correlate with migration and proliferation in breast cancer cells, was significantly down regulated in our microarray data in the indomethacin alone and full treatment groups compared to control. However protein analysis of CCN1 revealed that it was not significantly changed between

groups (Figure 26A). Also although S100A8 and S100A9 were highly altered in our RNA microarray data, protein levels of S100A8 and S100A9 were highly variable within our tumors and thus an exact correlation to our treatment regimes was not observed (Figure 26B). Levels of Osteoprotegerin (OPG) were significantly down regulated in vaccinated and full treatment mice compared to control (Figure 26C). Osteoprotegerin is a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL) which is a known activator of the immune system. Our treatment groups have decreases in the decoy receptor (OPG), which should lead to more activity of RANKL and thus an enhancement in inflammation which would help our treatments to be more effective. Finally we also observed increases in Arginase 1 levels in the Indomethacin and full treatment mice compared to controls (Figure 26A). Arginase metabolizes L-arginine to L-ornithine and urea, thus depleting arginine. In the field of tumor immunology depletion of L-arginine is known to suppress T cell immune responses and this has emerged as a fundamental mechanism of inflammation-associated immunosuppression. It is somewhat confusing as to what arginase is changing but it could be a result of an influx of macrophages to the area, which would coincide with the increases in MHC CII that we observed in our array data. We are finalizing immunohistochemical data measuring the influx of macrophages into the tumors. This recent data is currently being compiled and will be submitted to a peer-reviewed journal within the month.

RESEARCH ACCOMPLISHMENTS:

Previous Findings from year 1&2

We now have a better understanding of the role of IDO enzymatic activity on tumor development and immune functioning:

- ⤴ IDO expressing-MTAG tumors grow significantly larger than IDO null tumors in both the Blk6 and IDO-/- mice
- ⤴ Phenotype of the mouse does not affect tumor burden, ie. Tumor burden does not differ between tumors that were injected into IDO null mice or blk6 mice, no matter whether they were MTAG, IKOM, or MTAG.MUC1 tumors
- ⤴ IDO null tumors have significantly lower tumor burden than either of the two IDO producing tumors
- ⤴ The percentage of T-Regulatory cells are significantly increased in blk6 mice as compared to IDO null mice, no matter what cells were injected
- ⤴ The percentage of MDSCs are significantly higher when IKOM cells are injected into blk6 as compared to IDO-/- mice
- ⤴ The percentage of MDSCs are significantly lower when MTAG cells are injected into blk6 as compared to IDO-/-

We now have a better understanding of the role of IDO enzymatic activity on tumor development and immune functioning in MUC1 vaccinated mice:

- ⤴ We have generated and characterized the MTAG.MUC1 cell line for future use with continued experiments
- ⤴ We optimized an orthotopic injection animal model for use with the MTAG.MUC1 cell line, and now have an effective model to test vaccine combinations in.
- ⤴ We found that there was no enhanced efficacy of the MUC1 vaccine when it was combined with IDO inhibitor, 1-MT
 - However, we did find that there was an enhanced efficacy of the MUC1 vaccine when it was combined with the COX-1, COX-2 non-selective inhibitor, Indomethacin
- ⤴ PGEM levels were reduced in the tumor lysate of mice treated with the vaccine + indomethacin combination
- ⤴ However, we did find the MTAG.MUC1 tumor cells had significantly lower proliferative rates when treated with Indomethacin
- ⤴ We found that Indomethacin + vaccine combinational treatment was the most effective treatment in reducing tumor burden, and enhancing vaccine

Milestones accomplished in the training program include:

- ⤴ I have participated in the Tumor Immunology journal club
- ⤴ I have attended the weekly seminars at the Breast Health Center Program in the The Blumenthal Cancer Center
- ⤴ I passed my pre-qualifiers and qualifiers (March 2012, April 2012, respectively)
- ⤴ My thesis proposal was approved (April 2012)
- ⤴ I have completed the Advanced Immunology Course hosted by the American Association of Immunologists (July 2012)
- ⤴ I have attended workshops on How to Write a Competitive Grant Proposal (April 2012, October 2102)
- ⤴ I defended my dissertation work on February 21, 2013, and graduated May of 2013
- ⤴ We tested multiple drugs known to target the cyclooxygenase pathway to determine which drug would best enhance the efficacy of the MUC1 peptide vaccine in tumor bearing mice. Surprisingly, only Indomethacin, a non-steroidal anti-inflammatory drug, increased the efficacy of the MUC1 peptide vaccine. Since the combinational therapy significantly reduced tumor burden, we moved forward to investigate how indomethacin was enhancing the MUC1 peptide efficacy.
- ⤴ We did not observe enhancement of either MUC1 specific T cells responses (ELISPOT) or MUC1-specific antibody responses with combinational therapy compared to vaccine. Further no significant changes were observed in suppressor cells located in the tumor-draining lymph nodes of mice treated with the combinational therapy compared to the vaccine alone.
- ⤴ Immune cells isolated from the tumor-draining lymph nodes of vaccinating mice were unable to kill Mtag.MUC1 tumor cells in culture, indicating that these cells are resistant to immune killing. However, when Mtag.MUC1 tumor cells were pretreated with Indomethacin, immune cells were able to significantly reduce tumor cell proliferation and caused tumor cell death as measured by a chromium release assay. Further, Tunel staining was significantly increased in full treatment mice compared to control mice, indicating that this observation is occurring *in vivo*.
- ⤴ We also performed microarray analysis with tumors from either control mice or mice treated with indomethacin alone, vaccine alone, or combinational therapy. The full data set is attached in the file 'RNA microarray analysis'. Many markers related the innate inflammatory response were enhanced with full treatment, such as S100A8, S100A9, Fc receptors, MHC Class II molecules and even arginase were significantly upregulated. Also, osteoprotegerin (Tnfrsf11b) was significantly down regulated, which is an inhibitor of acquired tumor cell killing. Confirmatory Western blot analysis determined that arginase is increased in indomethacin alone or full treatment mice. It is somewhat confusing as to what arginase is changing but it could be a result of an influx of macrophages to the area, which would coincide with the increases in MHC CII that we observed in our array data. We are currently finalizing immunohistochemical data measuring the influx of macrophages into the tumors. Interestingly, we did observe a significantly down-regulating in osteoprotegerin (OPG) in vaccinated and full treatment mice compared to control. Osteoprotegerin is a decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL) which is a known activator of the immune system, and thus the down regulation of OPG in our full treatment mice could be response for the enhanced immune responses.

REPORTABLE OUTCOMES:

- We have generated a breast cancer cell line that was transfected with MUC1, to express human MUC1; designated MTAG.MUC1
- The research has been disseminated in two poster presentations at the American Association of Cancer Research
- We have optimized our orthotopic injection animal model for use with the MTAG.MUC1 cell line, and now have an effective model to test vaccine combinations in.
- We now have serum, tumor lysates, paraffin embedded tissue sections, tumors sections in RNA later and OCT frozen sections from tumor bearing mice, treated with a combination of vaccine + 1-MT, vaccine + celecoxib, vaccine + indomethacin, vaccine + AH6809, as well as control mice, and mice treated with indomethacin alone. We will use these repositories in the near future in a multiplex mouse cytokine array, and have already performed a microarray analysis with these samples.
- Obtained my PhD in Biology supported by this work
- Currently compiling all data for a Manuscript

CONCLUSION:

Treatments that work by modulating the immune response are amongst the most widely used and accepted medical treatments. Most efforts thus far in cancer immunotherapy have focused only on enhancing immunity. However, tumors create an abnormal local microenvironment that allows them to escape immune detection and destruction. Thus, immune evasion is one major obstacle that has to be addressed prior to designing and delivering successful immunotherapy. A landmark study by Munn et al. demonstrated that tumor cells utilize a system that contributes to the immune suppression via expression of IDO. This project is focused on 1). This project is focused on 1) understanding the role of IDO enzymatic activity on tumor development and immune function and 2) investigating the efficacy of a MUC1-based vaccine in combination with a variety of targeted inhibition of immune suppression in an effort to achieve a maximum clinical response and 3) Determining the mechanism behind the enhanced efficacy. Thus far, we have concluded that the phenotype of the mouse does not affect tumor burden. We found that tumor burden does not differ between tumors that were injected into IDO null mice or blk6 mice, no matter whether they were injected with IDO null or IDO expressing tumors. In this study, we generated a breast cancer cell line from the tumors of PyVMT mice and retrovirally infected the cells with the full length MUC1 plasmid. With the use of an orthotopic injectable model of breast cancer, we tested the MUC1 specific tumor vaccine in combination with four different drugs, each with targeted inhibition of immune suppression in an effort to achieve maximal vaccine efficacy. The results clearly indicated that, compared to vaccine alone, the only combinational therapy that significantly reduced tumor burden, was the combination of indomethacin + vaccine (Figure 17). This reduction in tumor burden was associated with a decrease in PGEM levels (Figure 36), indicating that indomethacin was indeed functional. The data clearly indicate that an enhanced vaccine efficacy can be achieved with a combination of MUC1 peptide vaccine + non-selective, COX-1 and COX-2 inhibitor, indomethacin. We performed a Microarray to further analyze the mechanism of enhanced efficacy as well as evaluated alterations in protein expression of CCN1, OPG, and Arginase. Our preclinical studies offer us an opportunity to assess the feasibility of inhibition of COX pathway in combination with immunotherapy for the treatment of breast cancer. With this information in mind, we can more effectively design a breast cancer vaccine that specifically targets the immunosuppressive agent that is most inhibitory to our vaccine treatment. The results of this study are currently being compiled for a manuscript submission.

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APPENDICES:

APPENDIX 1: AACR ABSTRACT SUBMISSION

AACR Annual Meeting 2012 in Chicago, IL

Temporary Abstract Number: 4153

Title: Investigating the role of IDO in MUC1 expressing breast cancers

Your above-referenced abstract has been scheduled for presentation in a Poster Session at the 2012 AACR Annual Meeting in Chicago, IL and will be published in the 2012 Proceedings of the American Association for Cancer Research. Presentation information pertaining to your abstract is below:

Session ID: Immunology 10

Session Date and Time: Wednesday Apr 4, 2012 8:00 AM - 12:00 PM

Location: Hall F, Poster Section 20

Permanent Abstract Number: 5400

Investigating the Role of IDO in MUC1 Expressing Breast Cancers

Short Title:

MUC1 and IDO in Breast Cancer

Author Block Dahlia Besmer, Amritha Kidiyoor, Sritama Nath, Lopamudra Das Roy, Jennifer Curry, Pinku Mukherjee.
Univ. of North Carolina at Charlotte, Charlotte, NC

Abstract:

Breast cancer is the second most common cancer in the U.S. and contributes to 40,000 deaths a year. Breast cancer vaccines are currently being considered as a clinical intervention that may reduce the chance of metastasis and recurrence, and perhaps even function to be effective in cancer prevention. MUC1, a membrane tethered mucin glycoprotein, is over expressed in >90% of breast cancers, and therefore has been recently described as the second most targetable tumor antigen by the National Cancer Institute. There are currently ongoing trials using the MUC1 vaccine in patients with metastatic breast cancer. However, immunotherapy has had limited success because tumors have the ability to undergo immune evasion tactics. This includes expression of Indoleamine 2,3-dioxygenase (IDO) immunosuppressive enzymes. IDO was first identified in maintaining maternal tolerance towards the antigenically foreign fetus during pregnancy. Its activity is increased under pathological conditions, including during tumor development. IDO is emerging as a key player in T cell suppression and in the induction of immune tolerance to tumors. The present study is focused on understanding the role of IDO enzymatic activity on tumor development and immune function. In this study, we injected two cell lines that express IDO, and one cell line that is IDO null (MTAG, MTAG.MUC1 and IKOM, respectively). These cell lines were injected into either IDO null mice, or control c57/bk6 mice (n=3 each). We hypothesized that IDO expression in the tumor microenvironment of mice creates a pathological state of immune suppression resulting in altered tumor progression and immune function. We show that mice (whether they be IDO null or blk6) injected with IKOM cells have rejected their tumors as compared to those injected with IDO expressing tumor cells ($p<0.05$). Mice injected into IDO null mice had significantly lower percentage of Tregulatory cells as compared to blk6 mice. Future studies would investigate the role of MUC1 based vaccines in combination with an IDO inhibitor, with the goal of reducing metastasis and increasing survival in patients with breast cancer.

APPENDIX 1: AACR ABSTRACT SUBMISSION

AACR Annual Meeting 2013 in Washington DC

Combinational MUC1 vaccine therapy and Indomethacin treatment reduces breast tumor burden via a COX-independent pathway.

Author Block Jennifer M Curry*, **Dahlia M Besmer***, Lopamudra D. Roy, Priyanka Grover, Sritama Nath, Shanti Rao, Pinku Mukherjee Univ. of North Carolina at Charlotte, Charlotte, NC

*Both authors contributed equally

Abstract:

While much advancement has been made in breast cancer treatment, metastatic breast cancer remains an incurable disease. MUC1 is a glycoprotein expressed on normal glandular epithelial but is over-expressed and underglycosylated in over 90% of human breast tumors and 100% of metastatic lesions, which lead to its ranking by NCI as the second most targetable antigen. Vaccines against tumor antigens have several benefits, including the chance to eliminate metastatic lesions that express the vaccinating tumor antigen. To this end, we have proposed vaccinating with peptides from the MUC1 protein core, which is only visible to the immune system on the tumor-associated form of the protein. Previous work from our lab has demonstrated that this vaccine does elicit a MUC1-specific immune response that can only be functional if the immunosuppressive tumor microenvironment is altered to allow efficient killing of tumor cells. Thus, we investigated the effectiveness of MUC1 vaccination in combination with drugs known to inhibit immunosuppression to determine which drug is the most effective. **Methods:** Mice that are transgenic for human MUC1 (MUC1.Tg) mice were orthotopically injected with a syngenic breast cancer cell line expressing human MUC1 (Mtag.MUC1). Mice were vaccinated after palpable tumor formation with the vaccine cocktail, consisting of two MHC class I-restricted MUC1 tandem repeat peptides and a class II pan helper peptide mixed with GM-CSF and CpG ODN, in incomplete Freund's adjuvant. Previous work in our lab has shown that blocking the cyclooxygenase pathway (COX) resulted in an inhibition of immunosuppression. Thus we used the following drugs in combination with the MUC1-vaccine therapy: Indomethacin (COX1 and COX2 inhibitor), Celecoxib (COX2 inhibitor), 1-methyl tryptophan (indoleamine 2,3 dioxygenase inhibitor), and AH6809 (EP2 receptor antagonist). Mice were euthanized and tissue was collected post the final vaccination. MUC1 vaccine therapy alone caused a slight reduction in tumor burden, although not significant. The combinational therapy of Indomethacin + Vaccine resulted in a significant reduction in tumor burden, whereas all other treatments resulted in no significant reduction in tumor burden, as measured by caliper measurements. The combination treatment of Vacc+Indomethacin and Vacc+Celecoxib both reduced PGE2 levels compared to vaccine alone. In a repeat experiment, we found that the combination of Vacc+Indomethacin caused a significant reduction in tumor wet weight compared to vaccine alone as well as compared to control. However, Indomethacin alone did not significantly reduce tumor wet weight compared to control, indicating a synergistic effect of vaccine and indomethacin. Since Indomethacin but not Celecoxib reduced tumor burden when given in combination with the MUC1 vaccine, we are further investigating COX-independent pathways involved in this mechanism.

SUPPORTING DATA

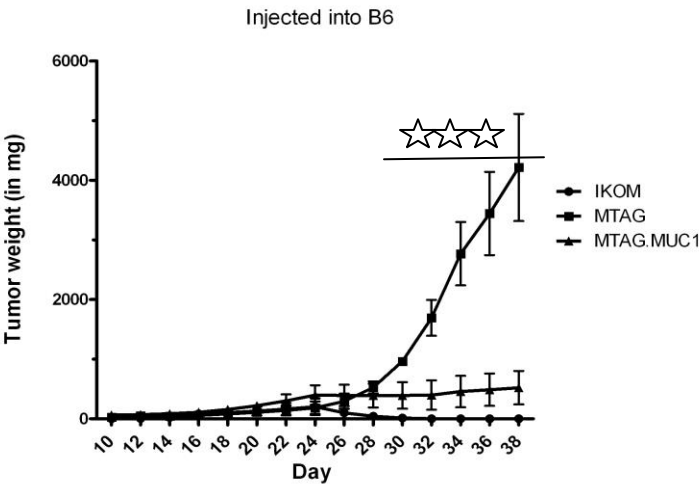
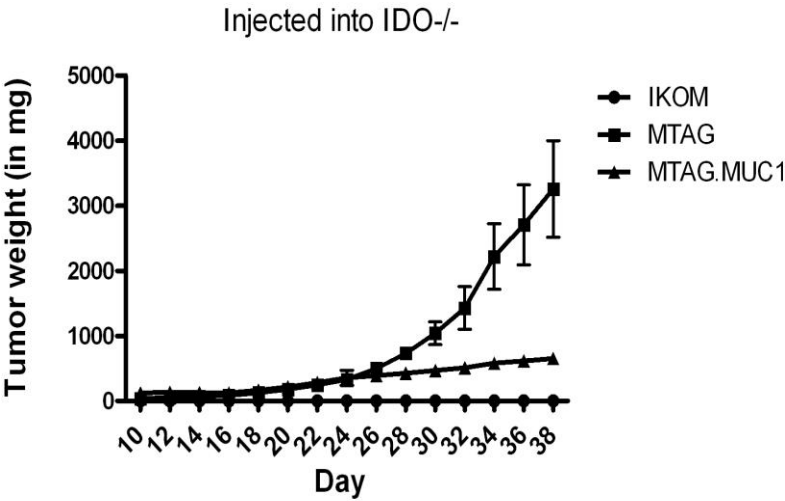


Figure 1: In the IDO-/- mice, MTAG and MTAG.MUC1 tumors grew rapidly, and again, IKOM tumors were rejected

Figure 2: In the Blk6 mice, MTAG and MTAG.MUC1 tumors grew rapidly, and again, IKOM tumors were rejected



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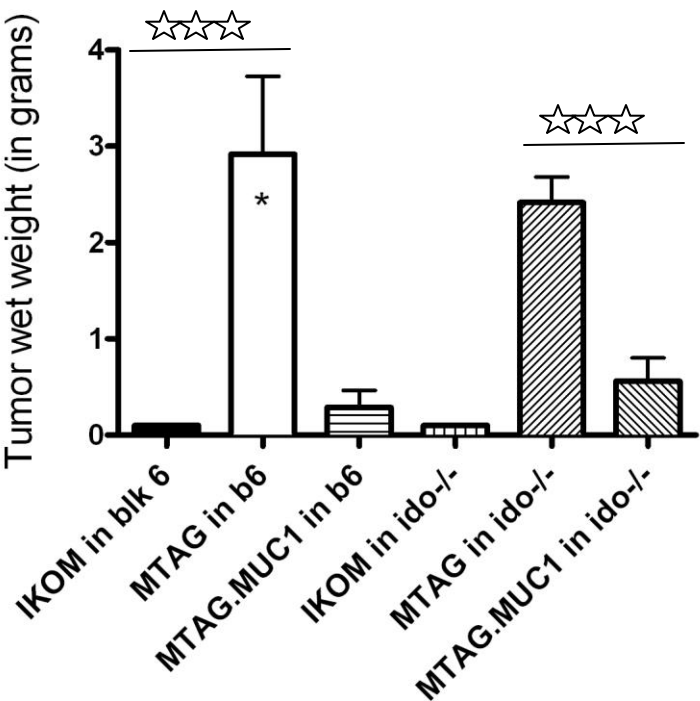


Figure 3: The tumor wet weight of MTAG tumors was significantly higher in the black6 mice as compared to both IKOM and MTAG.MUC1 tumors also injected into blk6 mice ($p<0.001$). Although there is a trend in which MTAG tumor size is decreased when injected into IDO-/- mice, there is no statistical significance. The tumor wet weight in the IDO-/- mice is similar in that MTAG tumor burden was significantly higher than MTAG.MUC1 and IKOM tumors. IKOM tumor burden, in both cases was significantly smaller than the IDO expressing tumors, MTAG, and MTAG.MUC1 ($p<0.01$).

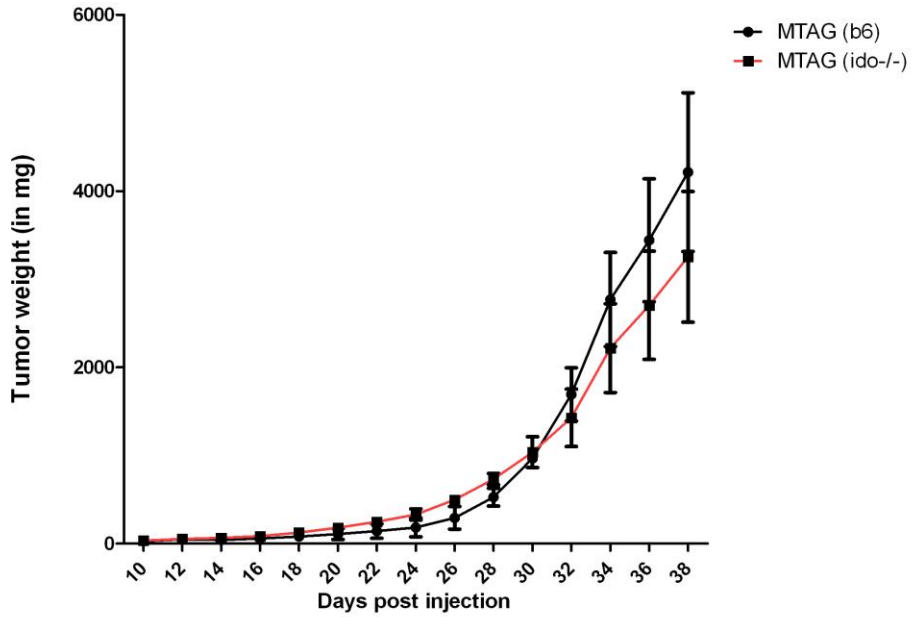


Figure 4: When comparing *IDO* expressing-MTAG tumors injected into *bk6* or *IDO*^{-/-} mice, there was no statistical significance in tumor burden, as assessed by caliper measurement.

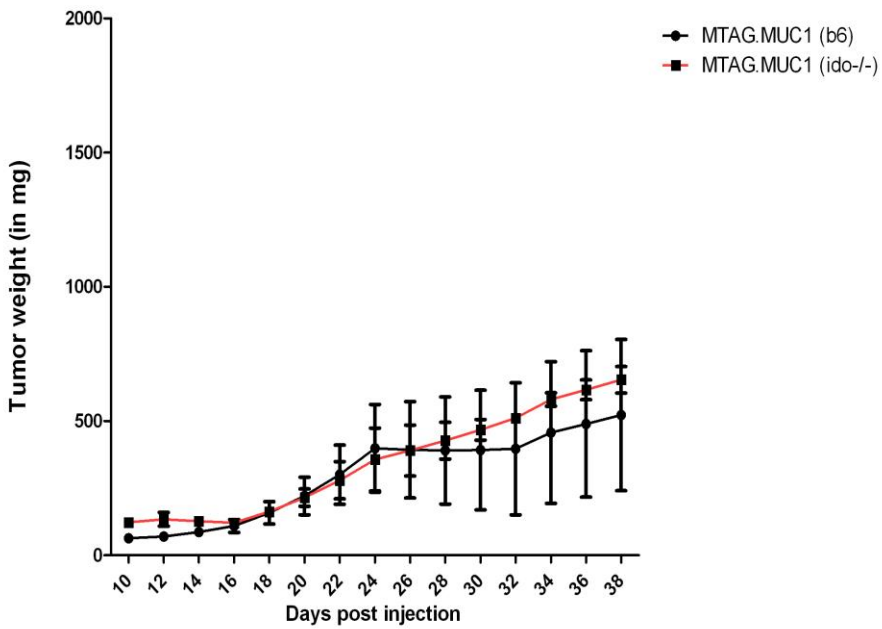


Figure 5: *IDO* expressing MTAG.MUC1 tumors injected into *blk6* or *IDO*^{-/-} mice are not statistically different

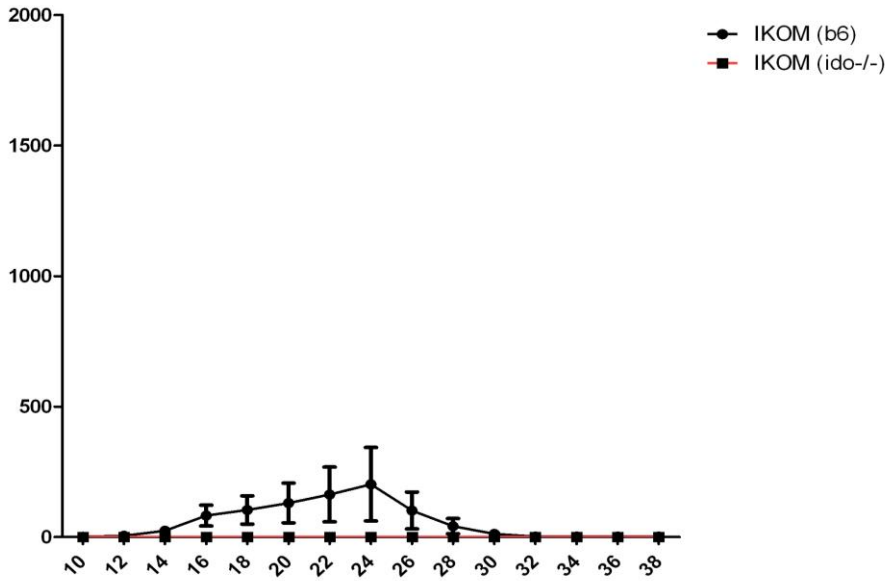


Figure 6: The IDO null cells injected into blk6 and IDO-/- were also not statistically different, although it is interesting to note that for the IKOM tumor cells, the tumors were starting to develop and progressed well until day 24, at which point, the NK cells, or T cells were able to clear the tumor, and reduce tumor burden to nothing

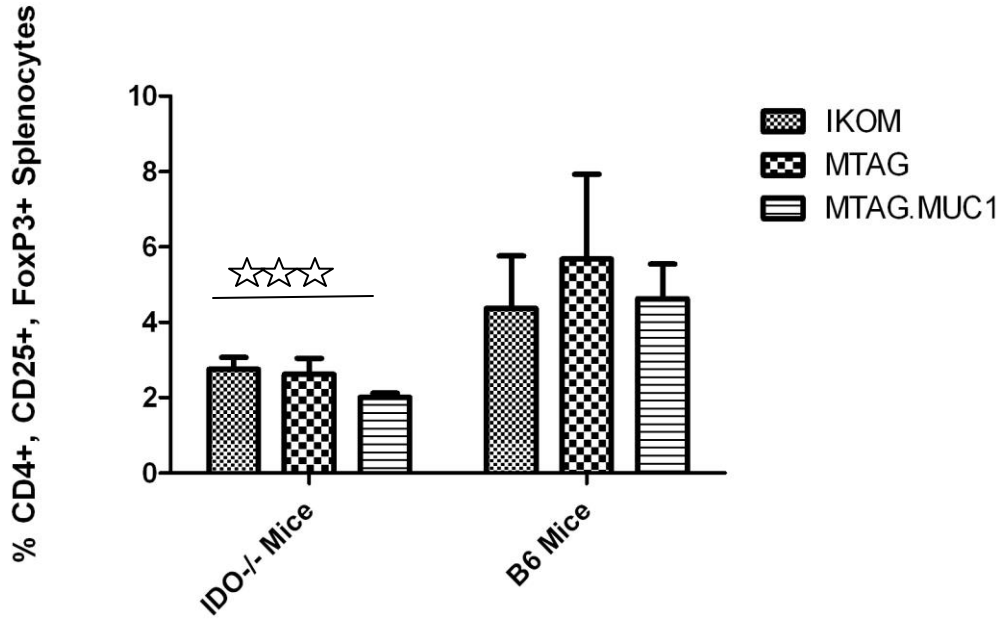


Figure 7: shows the percentage of $CD4^+$, $CD25^+$, $FoxP3^+$ Splenocytes (Tregs), in each of the IDO-/- and blk6 injected mice. The IDO-/- mice injected with tumor cells had significantly lower percentages of Tregs than the blk6 mice injected with the same tumor cells ($p < 0.05$)

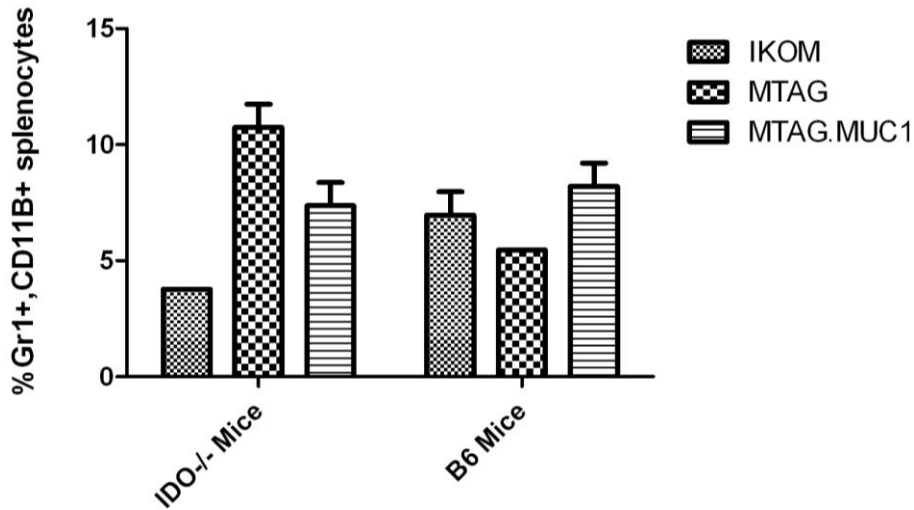


Figure 8: The percentage of MDSCs are significantly higher when IKOM cells are injected into blk6 as compared to IDO-/- mice

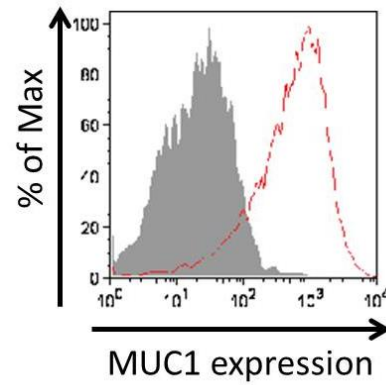


Figure 9: Characterization of the MTAG.MUC1 cell line. MUC1 expression was confirmed by flow cytometry. The gray histogram represents isotype control stained, and the red dashed line represents MUC1 staining.

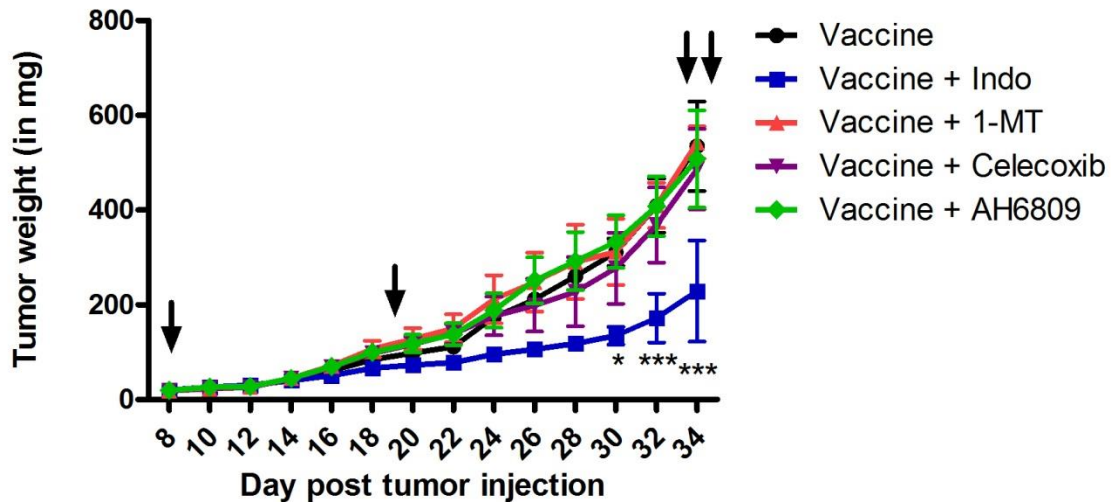


Figure 10: Indomethacin treatment with vaccination is the only combination that reduces tumor burden. Female MUC1.Tg mice, aged 8-12 weeks old were orthotopically injected with MTAG.MUC1 cells in the mammary fat pad (n=24). Tumors were palpable by day 8, and mice were randomly divided into 5 groups (n=5 per group, n=4 for vaccine). All mice were vaccinated on days 8, 19, 34, and 35 p.t.i.(as indicated by arrows) and treated with Celecoxib (10mg/kg), AH6809 (200ug), Indomethacin (3mg/kg) once daily, and 1-MT (400mg/kg) twice daily, five days a week. Tumor size was monitored by caliper measurements every other day until sacrifice. Body weight was measured every other day. Tumor weight was calculated according to the formula: grams = [(length in cm) x (width in cm)²]/2. Mice were sacrificed 35 days p.t.i, at which time, mice were not yet presenting with clinical signs indicating severe morbidity. Comparison of groups was done using a two-way ANOVA with a bonferoni post-hoc test (*, p<.05; **p<0.01; ***p<0.001 compared to control).

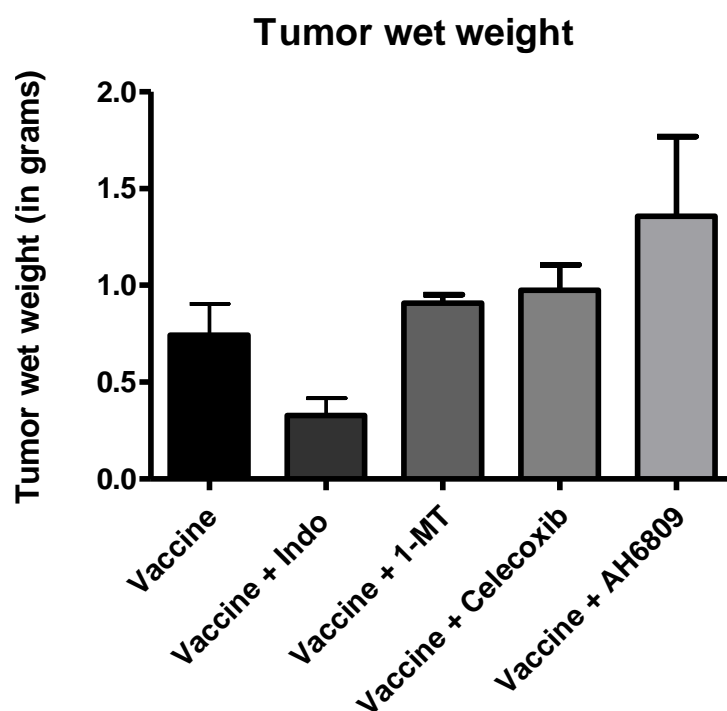


Figure 11: Indomethacin treatment with vaccination is the only combination that has a trend indicating reduced tumor burden. Female MUC1.Tg mice, aged 8-12 weeks old were orthotopically injected with MTAG.MUC1 cells in the mammary fat pad (n=24). Tumors were palpable by day 8, and mice were randomly divided into 5 groups (n=5 per group, n=4 for vaccine). All mice were vaccinated on days 8, 19, 34, and 35p.t.i. and treated with Celecoxib (10mg/kg), AH6809 (200ug), Indomethacin once daily (3mg/kg), and 1-MT (400mg/kg) twice daily, five days a week. Mice were sacrificed 35 days p.t.i, at which time tumors were excised and weighed. Comparison of groups was done using a one-way ANOVA with a Dunnetts multiple comparisons post hoc test. Although significance was not reached, there was a trend toward reduced tumor burden in the vaccine + indomethacin treatment group.

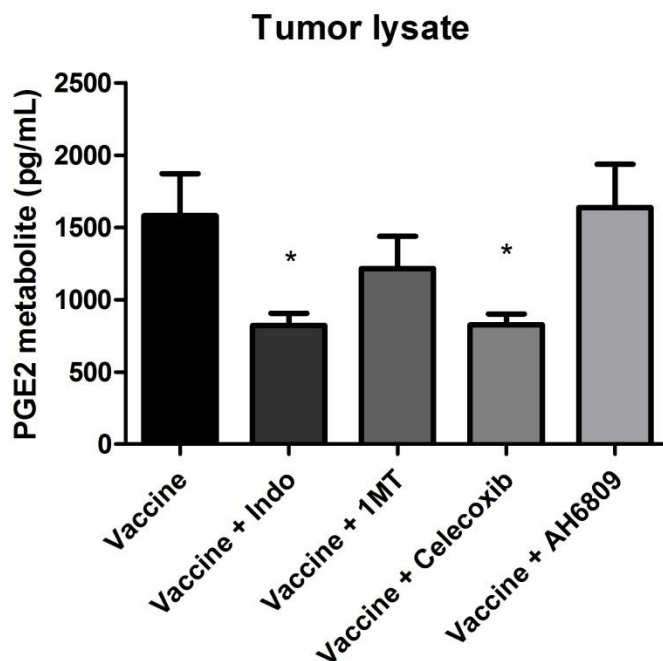


Figure 12: Celecoxib and Indomethacin both reduce PGE2 metabolite levels in combination with vaccination. Prostaglandin E2 Metabolite (PGEM) was measured in tumor lysate as a read out for PGE2 levels. Combinational treatment of vaccine + Indomethacin as well as vaccine + celecoxib significantly reduced tumor PGEM levels compared to vaccine treatment alone. Comparison of groups was done using a one-way ANOVA with a Dunnetts multiple comparisons post hoc test (*, $p < 0.05$ vs. vaccine alone).

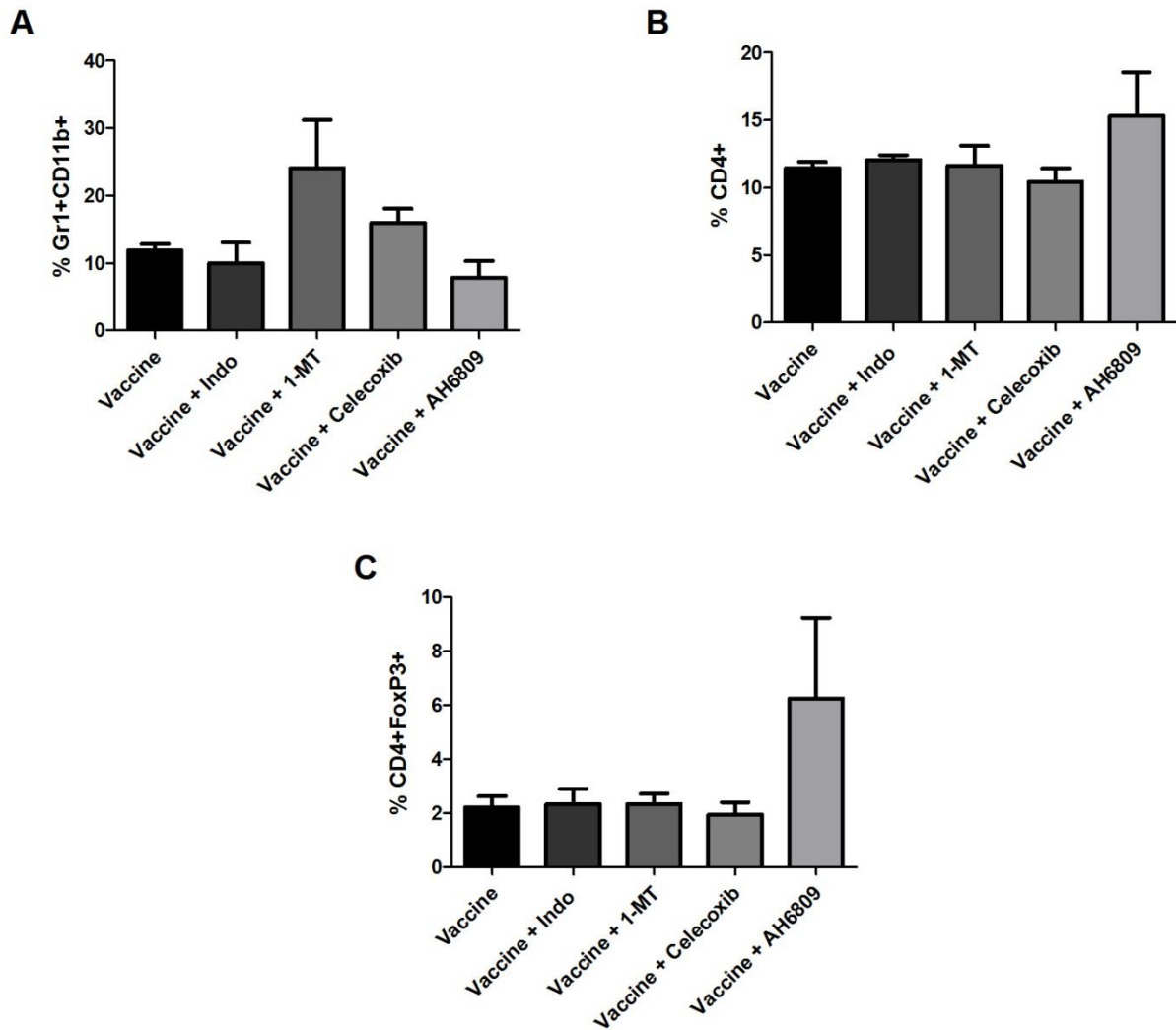


Figure 13: Immune analysis (MDSCs and Tregs) of combinational MUC1 vaccine therapy. Splenocytes from mice bearing MTAG.MUC1 tumors treated with vaccine therapy were assessed. A) Myeloid-derived suppressor cells (MDSCs) were characterized as Gr1+CD11b+ splenocytes. There was no significant difference in MDSC levels in mice treated with any of the combinational treatments. Vaccine in combination with 1-MT was the only group that seemed to increase MDSC levels, although the increase was not significant. B) Helper T cells were defined as CD4+ splenocytes. No significant difference was observed in the levels of T helper cells in any of the combinational treatment groups. C) Levels of T regulatory cells were measured in splenocytes, as defined by the co-expression of CD4 and FoxP3. No significant difference was observed in the levels of T regulatory cells in any of the treatment groups; however, the combination of Vaccine+AH6809 seems to increase percentage of T regulatory cells, although this increase was not significant. Comparison of groups was done using a one-way ANOVA with a Dunnetts multiple comparisons post hoc test (*, $p < 0.05$ vs. vaccine alone).

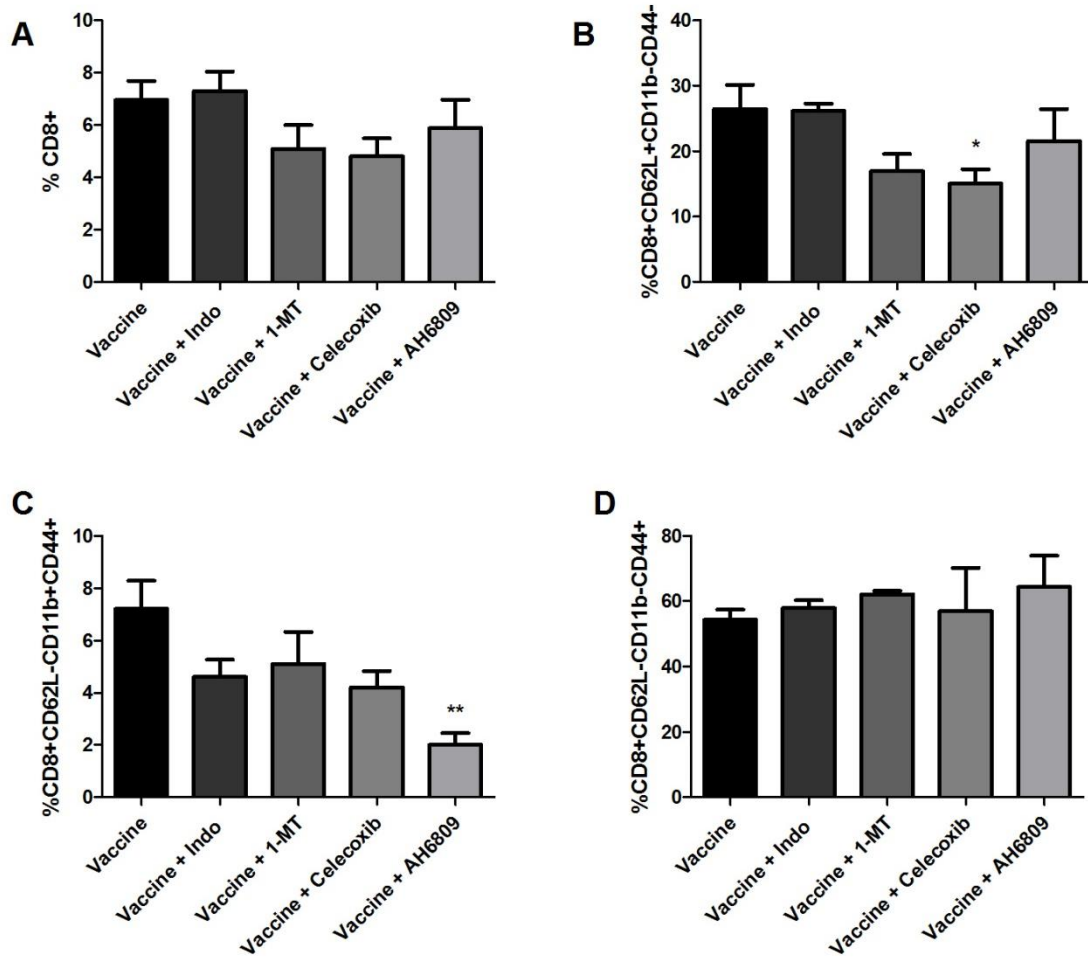


Figure 14: Immune analysis (T cells) of combinational MUC1 vaccine therapy.

Splenocytes from MTAG.MUC1 tumor bearing mice treated with MUC1 vaccine therapy were analyzed for T cell flow panels. For the T cell panel, Naïve T cells were defined as CD8+CD62L+CD11b-CD44-, Effector T cells were defined as CD8+CD62L-CD11b+CD44+ and Memory T cells were defined as CD8+CD62L-CD11b-CD44+. A) No significant changes were observed among the different treatment groups in overall CD8+ T cells. B) The combinational treatment of Vaccine+Celecoxib significantly reduced levels of Naïve T cell populations. C) The combinational treatment of Vaccine+AH6809 significantly decreased the percentage of effector T cells. D) No significant changes were observed among the different treatment groups in reference to memory T cells. Comparison of groups was done using a one-way ANOVA with a Dunnetts multiple comparisons post hoc test (*, $p < 0.05$, **, $p < 0.01$ vs. vaccine alone).

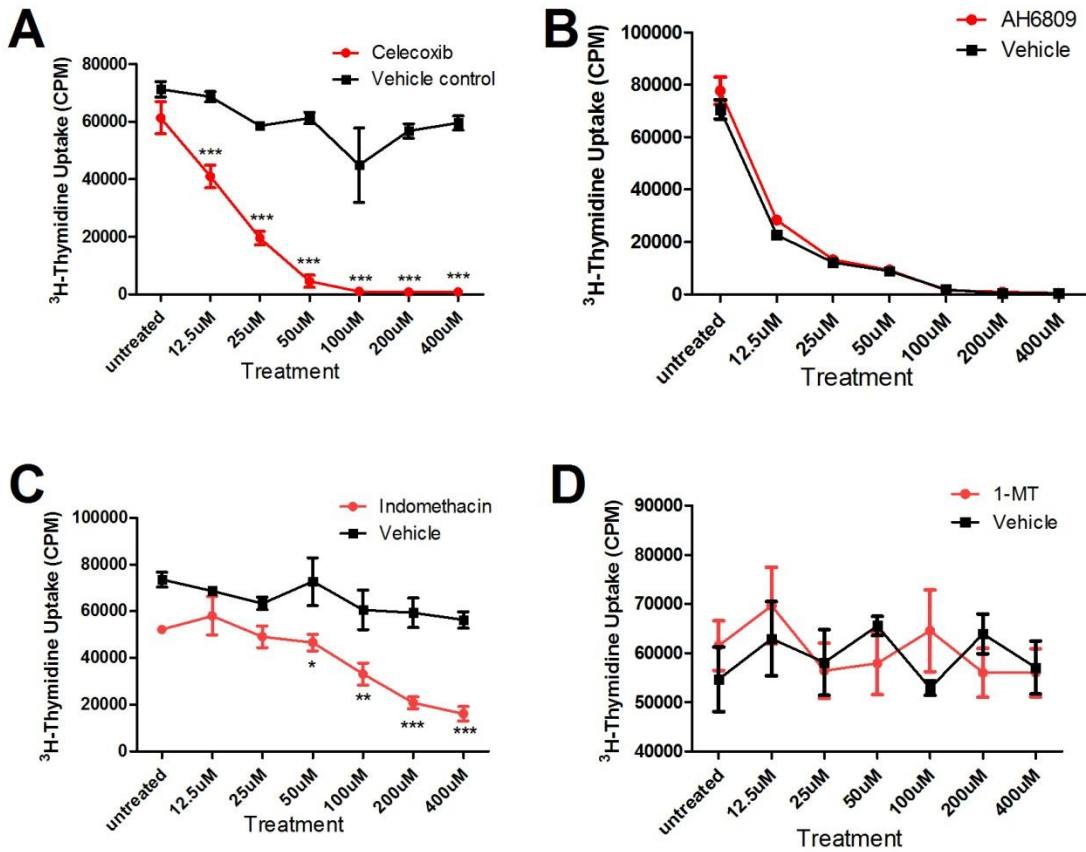


Figure 15: Proliferation assessed at 24 hours post treatment. Proliferation was measured by [3 H]-thymidine uptake. A) Treatment of MTAG.MUC1 cells with Celecoxib resulted in a significant decrease in proliferation at all dosages tested. B) There was no significant difference in proliferation of MTAG.MUC1 cells treated with AH6809. C) A significant decrease in proliferation of MTAG.MUC1 cells was noted when cells were treated with 50, 100, 200, and 400uM Indomethacin. D) No significant difference was observed when cells were treated with varying doses of 1-MT. Comparison of groups was done using a two-way ANOVA with a Bonferoni post hoc test (*, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$ vs. vehicle alone).

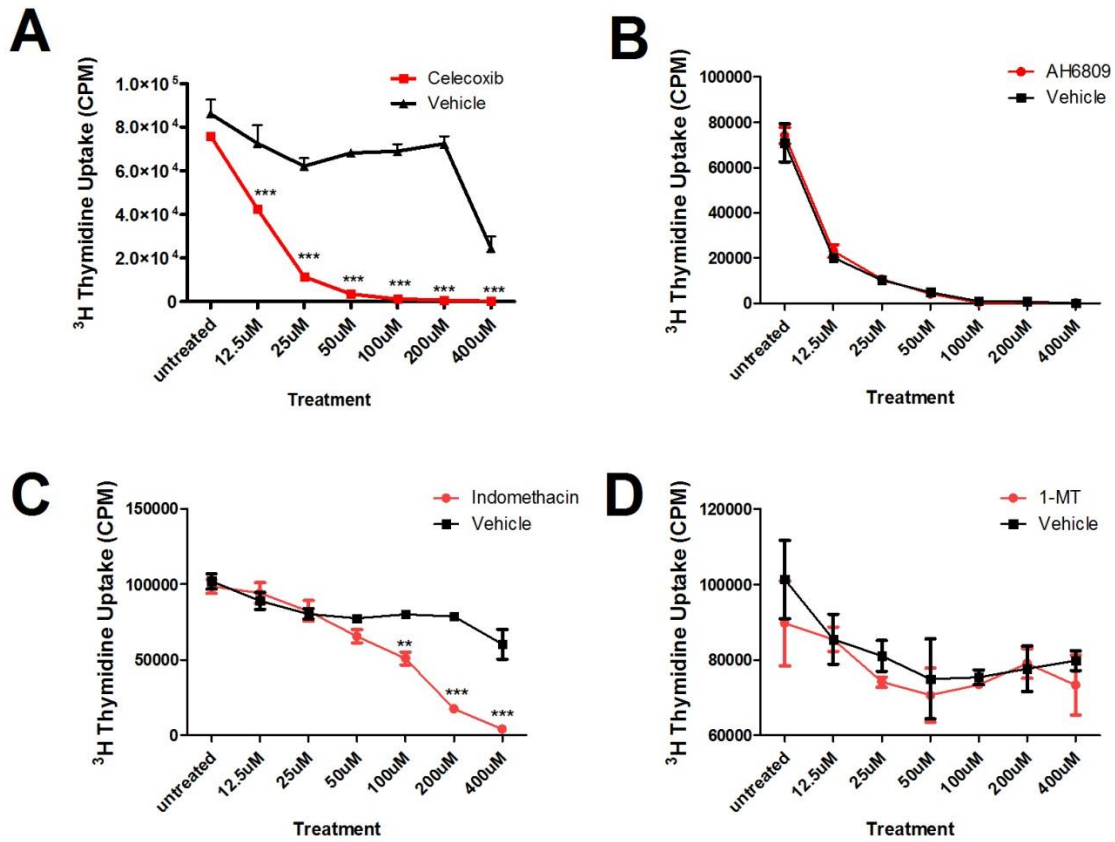


Figure 16: Proliferation assessed at 48 hours post treatment. Proliferation was measured by [³H]-thymidine uptake. A) Treatment of MTAG.MUC1 cells with Celecoxib resulted in a significant decrease in proliferation at all dosages tested. B) There was no significant difference in proliferation of MTAG.MUC1 cells treated with AH6809. C) A significant decrease in proliferation of MTAG.MUC1 cells was noted when cells were treated with 100,200, and 400uM Indomethacin. D) No significant difference was observed when cells were treated with varying doses of 1-MT. Comparison of groups was done using a two-way ANOVA with a Bonferoni post hoc test (*, p<0.05, **, p<0.01, ***, p<0.001 vs. vehicle alone).

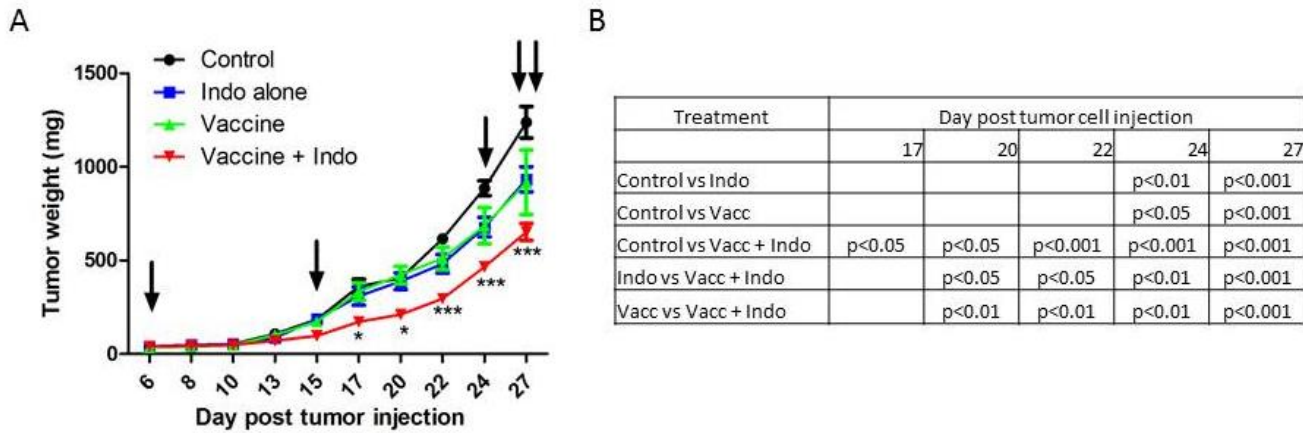


Figure 17: Combinational treatment of Vaccine + Indomethacin significantly reduces tumor burden. Female MUC1.Tg mice, aged 8-12 weeks old were orthotopically injected with MTAG.MUC1 cells in the mammary fat pad (n=23). Tumors were palpable by day 6, and mice were randomly divided into 4 groups (n=6 per group, n=5 for indomethacin alone). One group served as a control, the indomethacin group was gavaged daily with 3mg/kg. The vaccine groups were vaccinated on days 6, 15, 24, 27, and 28 (as indicated by arrows). The combinational treatment group received both vaccination as well as three times a week treatment of indomethacin (3mg/kg) by gavage. Tumor size was monitored by caliper measurements three times a week, and body weight was measured twice weekly. Tumor weight was calculated according to the formula: grams = [(length in cm) x (width in cm)²]/2. Mice were sacrificed on day 27 and 28 days p.t.i. A) Treatment with vaccine + indomethacin resulted in a significant decrease in tumor burden vs. control beginning at day 17. B) Table displaying significant decreases in tumor burden. Data were analyzed using GraphPad software and are expressed as mean \pm standard error mean. Comparison of groups was done by two-way ANOVA (*p<0.05, **p<0.01, ***p<0.001).

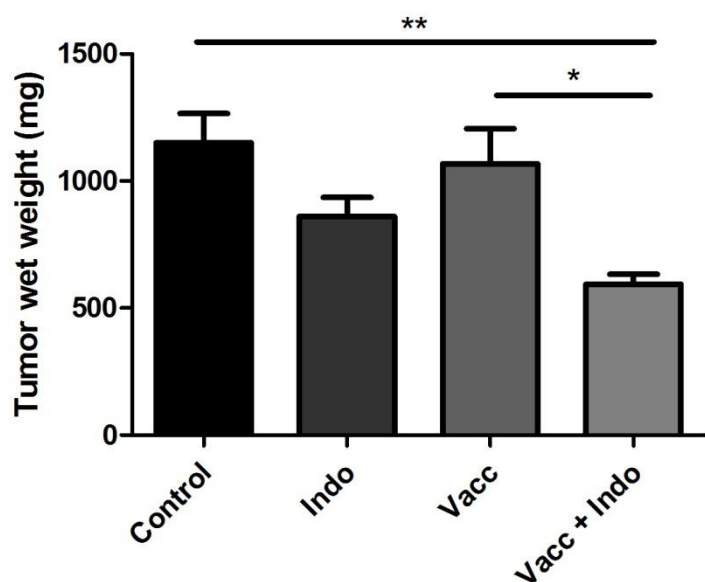


Figure 18: Combinational treatment of Vaccine + Indomethacin significantly reduces tumor wet weight. Female MUC1.Tg mice, aged 8-12 weeks old were orthotopically injected with MTAG.MUC1 cells in the mammary fat pad (n=23). Tumors were palpable by day 6, and mice were randomly divided into 4 groups (n=6 per group, n=5 for indomethacin alone). One group served as a control, the indomethacin group was gavaged daily with 3mg/kg. The vaccine groups were vaccinated on days 6, 15, 24, 27, and 28. The combinational treatment group received both vaccination as well as three times a week treatment of indomethacin (3mg/kg) by gavage. Mice were sacrificed on day 27 and 28 days p.t.i. Tumors were excised and weighed. Mice receiving the combinational treatment vaccine+indomethacin had significantly reduced tumor wet weight as compared to vaccine alone as well as control. Data were analyzed using GraphPad software and are expressed as mean \pm standard error mean. Comparison of groups was done by one-way ANOVA with Tukey's post hoc test (* p <0.05, ** p <0.01, *** p <0.001).

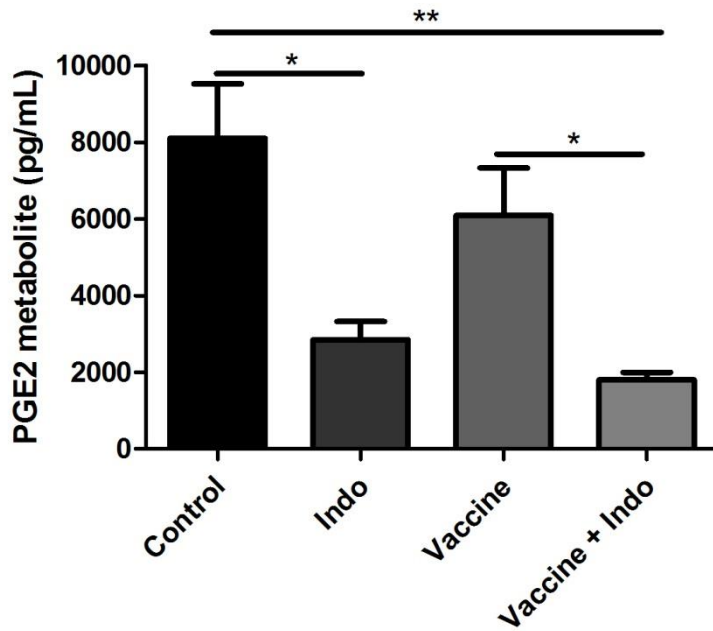


Figure 19: Indomethacin reduces PGE2 metabolite levels alone and in combination with vaccination. Prostaglandin E2 Metabolite (PGEM) was measured in tumor lysate as a read out for PGE2 levels. Indomethacin alone as well as the combinational treatment of vaccine + Indomethacin significantly reduced tumor PGEM levels compared to control. Additionally, the combinational treatment resulted in significantly reduced tumor PGEM levels as compared to vaccine alone. Comparison of groups was done using a one-way ANOVA with a Tukey's multiple comparisons post hoc test (*, $p < 0.05$ vs. vaccine alone)

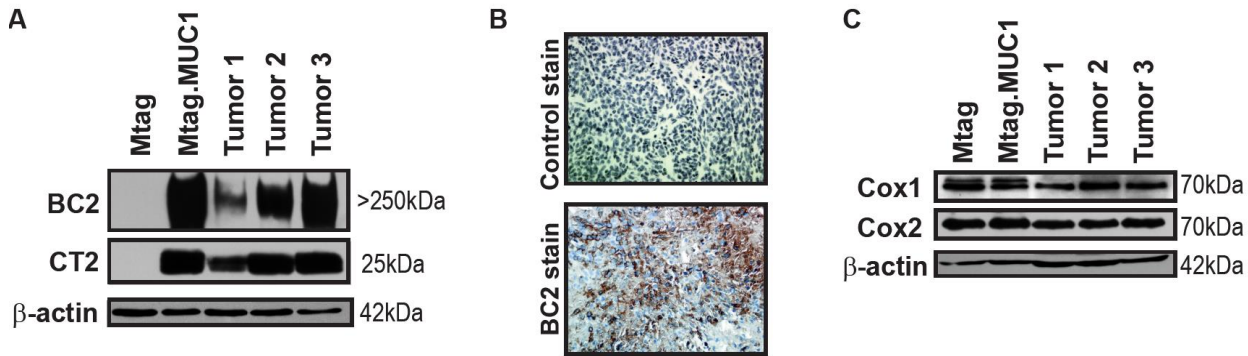


Figure 20: Mtag.MUC1 tumors express high levels of human MUC1, COX-1 and COX-2 in vitro and in vivo. A) Mtag cells were transfected to express the full length human MUC1 protein. Extracellular MUC1 (MUC1-EC) and the cytoplasmic tail of MUC1 (MUC1-CT) are detectable on the Mtag.MUC1 cell line in vitro and on tumors formed in MUC1 transgenic mice. B) MUC1.Tg mice were injected with 1×10^6 Mtag.MUC1 cells in the mammary fat pad. After 27 days, tumors were removed, formalin-fixed, paraffin, embedded, sectioned and stained with either an isotype control or the TAC 004 antibody. We confirm expression of MUC1 in our tumors generated from Mtag.MUC1 cells. C) We confirm Cox1 and Cox2 expression in the Mtag.MUC1 cell line and tumors.

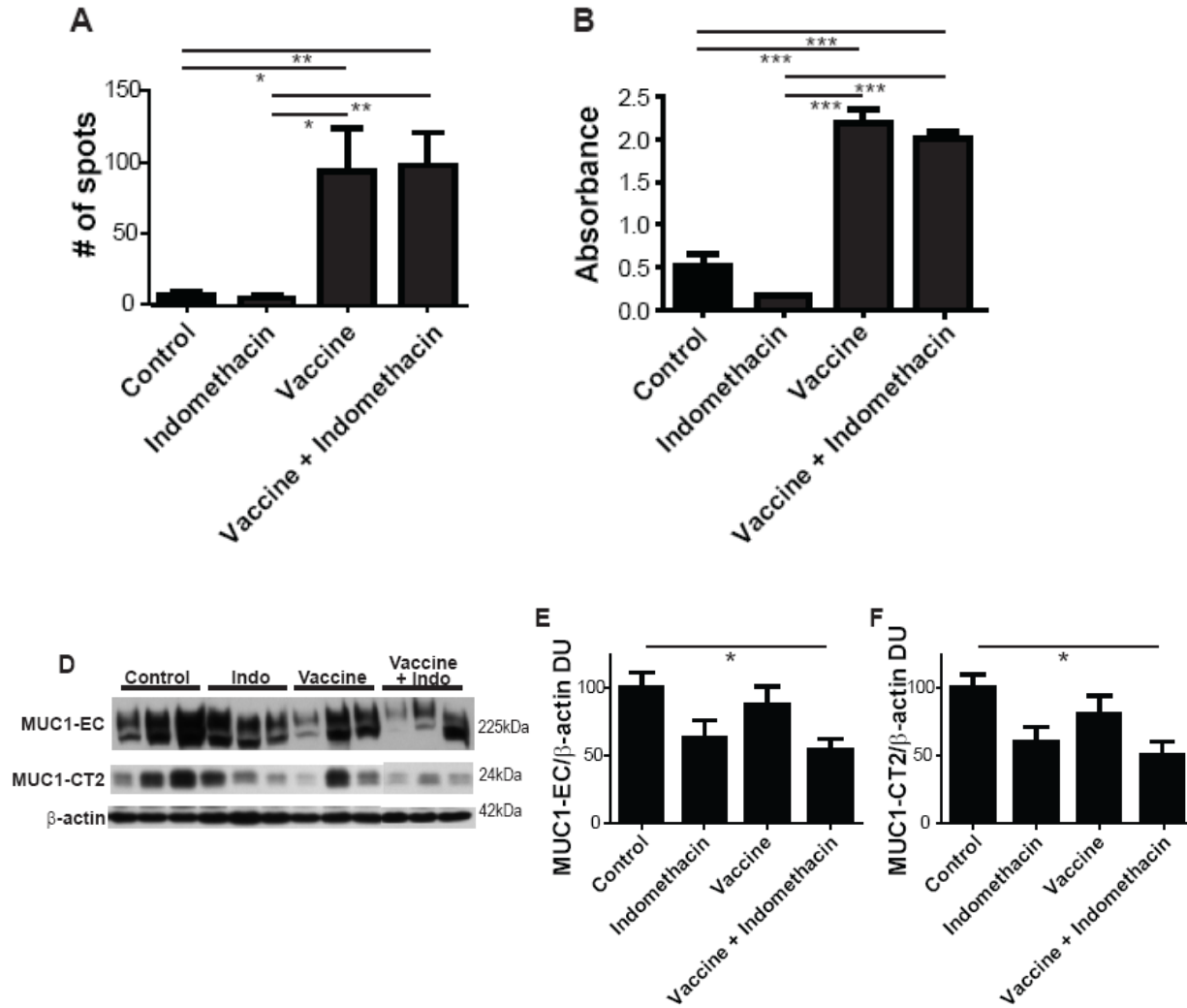


Figure 21: Immunological and tumor analysis of mice treated with the MUC1 peptide vaccine and Indomethacin. **A)** Tumor-draining lymph nodes were assessed for IFN γ production in response to vaccinating peptides. Both groups of vaccinated mice produced more IFN γ compared to control mice or mice treated with Indomethacin alone, but no difference was observed between vaccinated mice and combinational therapy mice. **B)** MUC1 antibody response was assessed via ELISA to the TAPPA peptide. Both groups of vaccinated mice produced more MUC1 antibody compared to control mice or mice treated with Indomethacin alone, but no difference was observed between vaccinated mice and combinational therapy mice. **C-E)** Levels of intra-tumoral MUC1 were assessed using the BC2 antibody (extra-cellular MUC1) and CT2 antibody (cytoplasmic tail). Intra-tumoral levels of MUC1 were significantly decreased in mice treated both the MUC1 peptide vaccine and Indomethacin compared to control mice. Pixels of MUC1 were normalized to β -actin and then control mice were set to 100% MUC1 expression. Comparison of groups was done by one-way ANOVA with Tukey's post hoc test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

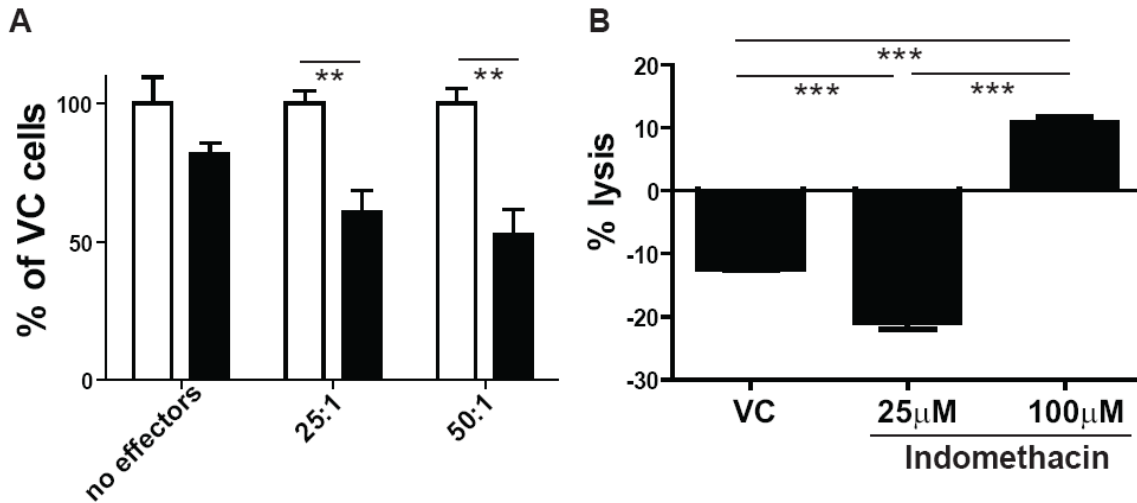


Figure 22: Immunological and tumor analysis of mice treated with the MUC1 peptide vaccine and Indomethacin.

A) Mtag.MUC1 cells were treated with 400 μ M Indomethacin for 24hrs. Then effector cells isolated from the tumor-draining lymph node of mice treated with the MUC1 peptide vaccine were co-cultured with Mtag.MUC1 cells at 50:1 and 25:1 effector:target ratios. H3 was added to the culture for 24hrs, when the plates were then washed to removed the lymphocytes and then incorporated thymidine was evaluated by using the Topcount microscintillation counter. Counts per minute were normalized to vehicle control levels. Effector cells were able to significantly inhibit proliferation in indomethacine treated Mtag.MUC1 cells (black bar) compared to vehicle treated cells (white bar). Comparisions between groups was conducted using a two-way ANOVA with a Bonferroni post-hoc test (** $p < 0.01$). **B)** Mag.MUC1 tumor cells were pretreated with 25 and 100 μ M of Indomethacin for 24hrs and then labeled with Cr51. Effectors cells from the tumor-draining lymph nodes of vaccinated mice were co-cultured with Cr51 labeled tumor cells at 100:1 ratio and chromium release was measured after 4hrs. Percent lysis was calculated using the following formula: $(\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm}) * 100$. Effector cells were only able to kill tumor cells when they were pretreated with 100 μ M of Indomethacin. Comparison between groups was conducted using a one-way ANOVA with Tukey's multiple comparison test (** $p < 0.001$).

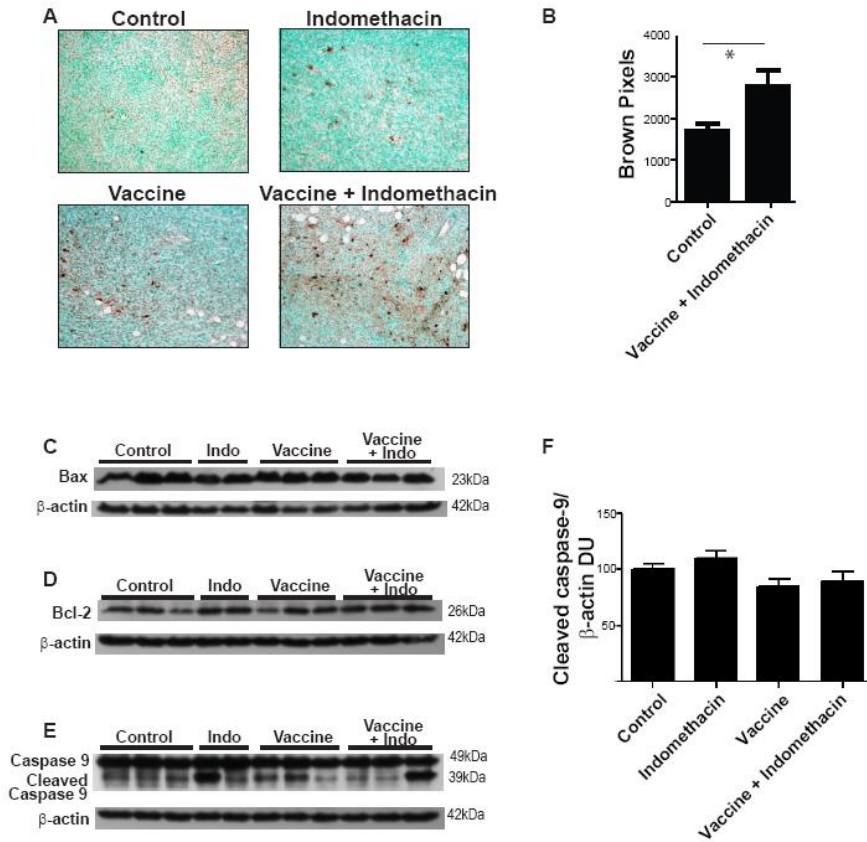


Figure 23: Assessment of apoptosis occurring within the tumors of mice treated with Indomethacin, the MUC1 peptide vaccine or combinational therapy. A and B) Apoptotic cells within the tumors of treated mice were assessed via TUNEL stain and brown stain was quantified using Adobe Photoshop. Student's T-test was used to compare control vs full treatment mice. **C)** Levels of the proapoptotic protein, Bax, were measured on tumor lysate via Western blot. Pixels of Bax were normalized to β -actin and then control mice were set to 100% Bax expression. **D)** Levels of the anti-apoptotic protein, Bcl-2, were measured on tumor lysate via Western blot. Pixels of Bcl-2 were normalized to β -actin and then control mice were set to 100% Bcl-2 expression. **E and F)** Levels of Caspase9 and cleaved Caspase9, indicating the activated form, were measured on tumor lysate via Western blot. Pixels of cleaved caspase-9 were normalized to β -actin and then control mice were set to 100% cleaved caspase-9 expression. Comparison of groups was done by one-way ANOVA with Tukey's post hoc test (n=6 for control, vaccine and vaccine + indo groups and n=5 for indomethacin alone group).

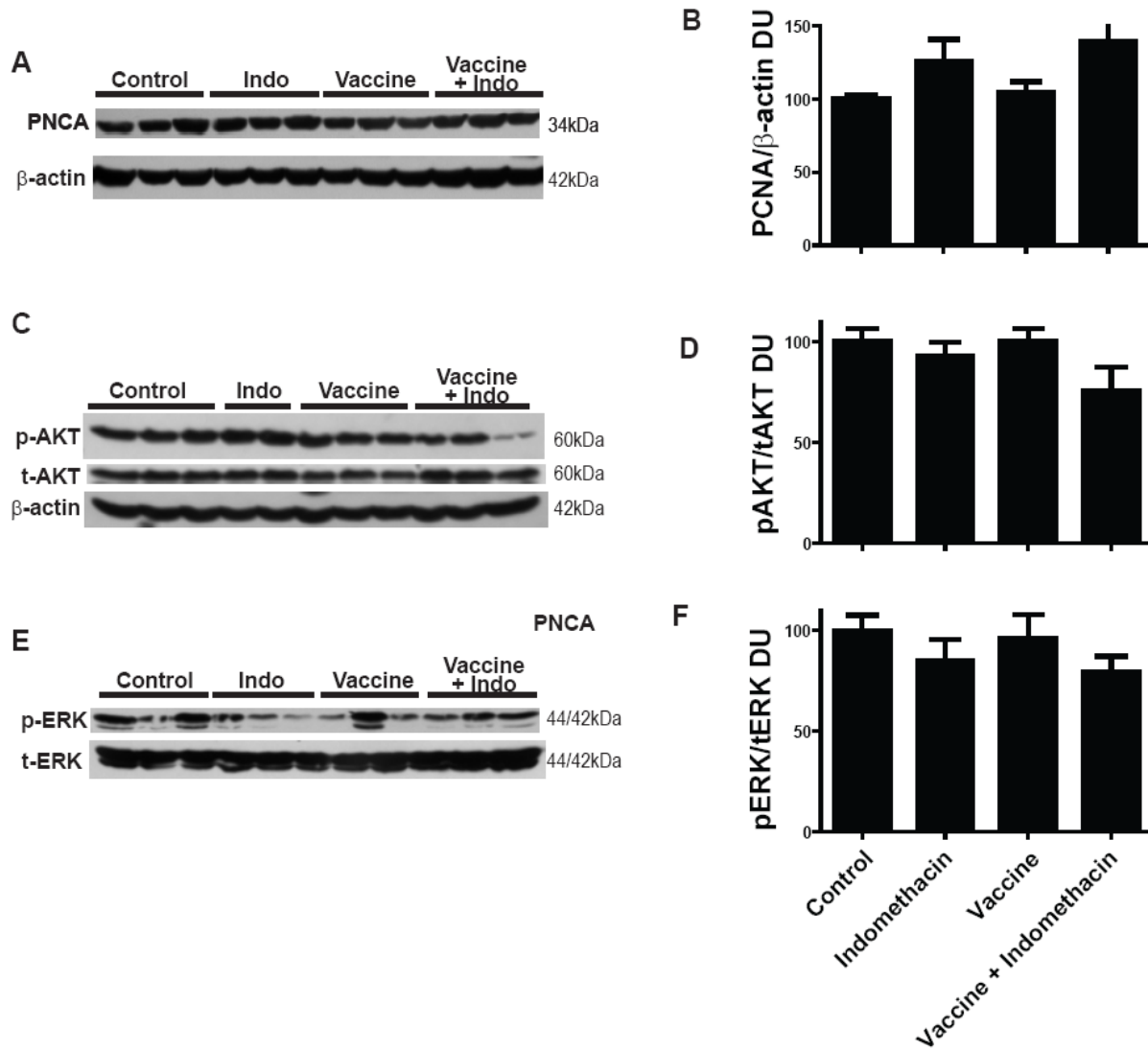


Figure 24: Levels of intratumoral proliferation markers are unchanged with Indomethacin and MUC1 vaccine treated mice. A and B) Levels of PCNA was assessed on tumor lysate taken from mice in Figure 21. Pixels of PCNA were normalized to β -actin and then control mice were set to 100% PCNA expression. **C and D)** Levels of phosphorylated AKT (p-AKT) and total AKT (t-AKT) were measured on tumor lysate taken from mice in Figure 21. Pixels of p-AKT were normalized to t-AKT and then control mice were set to 100% pAKT expression. **E and F)** Levels of phosphorylated ERK (p-ERK) and total ERK (t-ERK) were measured on tumor lysate taken from mice in Figure 21. Pixels of p-ERK were normalized to t-ERK and then control mice were set to 100% p-ERK expression. Comparison of groups was done by one-way ANOVA with Tukey's post hoc test.

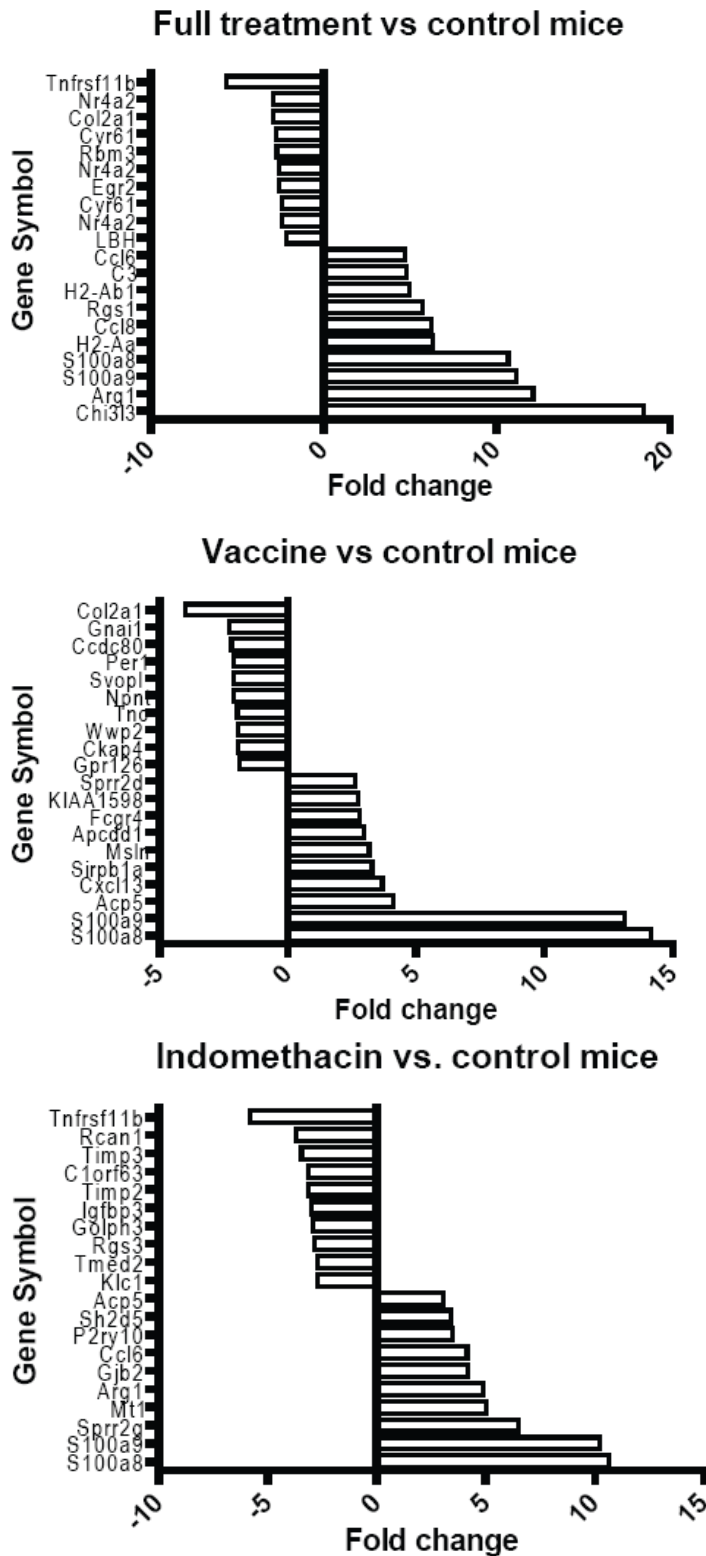


Figure 25: Microarray analysis of tumors from mice treated with Indomethacin, the MUC1 peptide vaccine or a combination of the two therapies. RNA was isolated from Mtag.MUC1 tumors from mice treated with either the vaccine, indomethacin or combinational therapy. Microarray analysis was conducted and the top ten significantly upregulated and downregulated genes from the treatment groups compared to untreated mice are displayed (n=2 per group, $p > 0.05$ was considered significant).

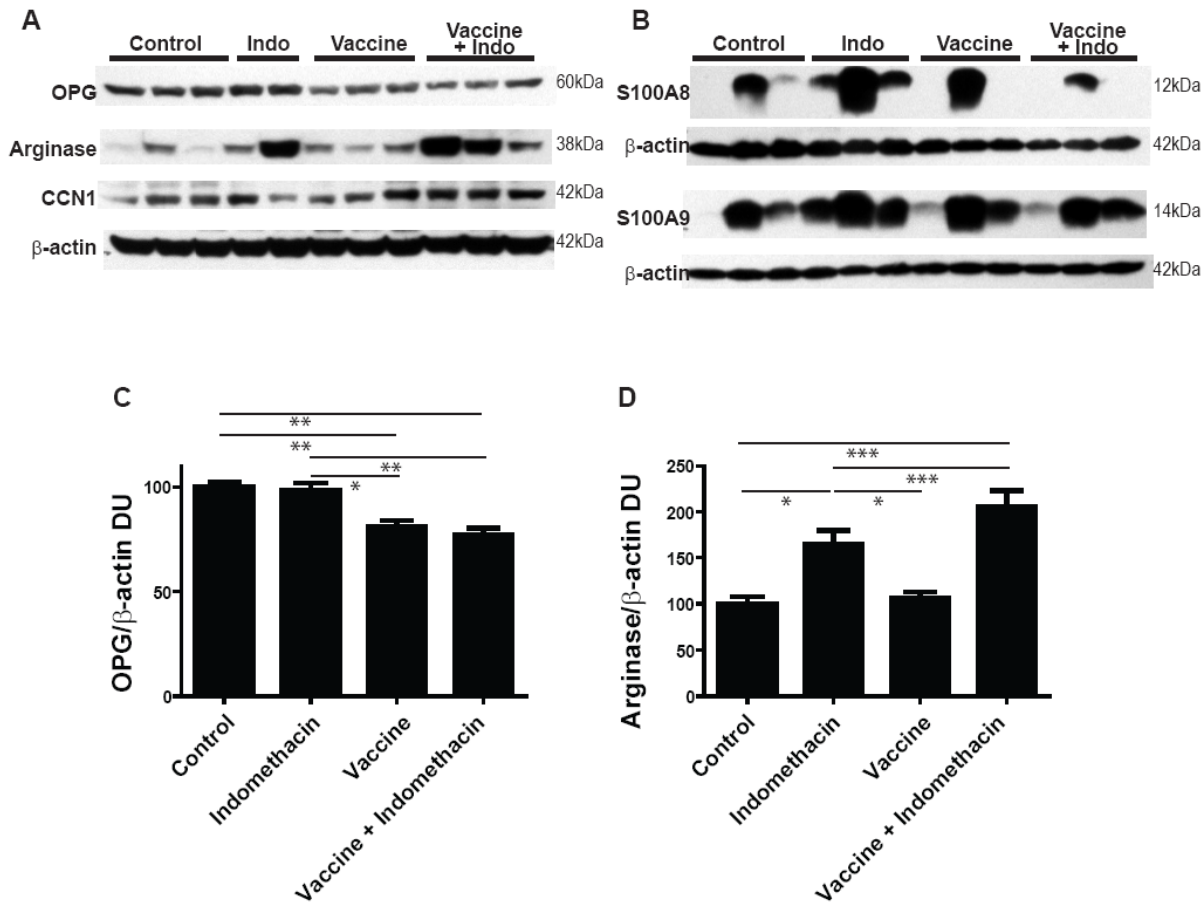


Figure 26: Combinational therapy with indomethacin and MUC1 peptide vaccine significantly reduces osteoprotegerin (Tnfrsf11b/OPG) and significantly increase arginase within the tumors of mice. Genes determined to be altered via the RNA microarray were assessed for protein differences in the tumor lysates of mice treated with indomethacin, vaccine or combinational therapy. **A, C and D)** OPG was significantly reduced in vaccinated and full treatment groups compared to control. Levels of the CCN1 protein were unaltered by treatment. **B)** When assessed at a protein level, S100A8 or S100A9 were unchanged with treatment, indicating that they are not the mechanism of action. Comparison of groups was done by one-way ANOVA with Tukey's post hoc test (n=6 for control, vaccine and vaccine + indo groups and n=5 for indomethacin alone group) (*p<0.05, **p<0.01, ***p<0.001).